

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

7933-38

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

Not Known 097744406

INTERNATIONAL APPLICATION NO.
PCT/US99/15594INTERNATIONAL FILING DATE
July 8, 1999PRIORITY DATE CLAIMED
July 24, 1998

TITLE OF INVENTION

Allogeneic Cellular Immunogens Useful As Cancer Vaccines

APPLICANT(S) FOR DO/EO/US

Michael S. Halpern and James M. England

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
- ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
- ☒ A copy of the International Search Report (PCT/ISA/210).
- ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
- ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
- ☒ A copy of the International Preliminary Examination Report (PCT/PEA/409).
- ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

10. The Declaration is two separately executed documents.

Small Entity Status is claimed

Prepaid Return Receipt Postcard

| | | |
|---|--|--|
| U.S. APPLICATION NO. 09/77444 Not Known Yet | INTERNATIONAL APPLICATION NO. PCT/US99/15594 | ATTORNEY'S DOCKET NUMBER 7933-38 |
|---|--|--|

| | | | | | |
|--|---------------------|---------------------|-------------|----------------------------------|-----------|
| 21. The following fees are submitted: | | | | CALCULATIONS PTO USE ONLY | |
| BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : | | | | | |
| <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO | | | | \$970.00 | |
| <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO | | | | \$840.00 | |
| <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO | | | | \$690.00 | |
| <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) | | | | \$670.00 | |
| <input checked="" type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) | | | | \$96.00 | |
| ENTER APPROPRIATE BASIC FEE AMOUNT = | | | | \$50.00 | |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). | | | | \$0.00 | |
| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
| Total claims | 28 - 20 = | 8 | x \$9.00 | \$72.00 | |
| Independent claims | 4 - 3 = | 1 | x \$40.00 | \$40.00 | |
| Multiple Dependent Claims (check if applicable) <input type="checkbox"/> | | | | \$0.00 | |
| TOTAL OF ABOVE CALCULATIONS = | | | | \$162.00 | |
| Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable) <input type="checkbox"/> | | | | \$0.00 | |
| SUBTOTAL = | | | | \$162.00 | |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). | | | | \$0.00 | |
| TOTAL NATIONAL FEE = | | | | \$162.00 | |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable) <input type="checkbox"/> | | | | \$0.00 | |
| TOTAL FEES ENCLOSED = | | | | \$162.00 | |
| | | | | Amount to be refunded | \$ |
| | | | | charged | \$ |

- ☐ A check in the amount of _____ to cover the above fees is enclosed.
- ☒ Please charge my Deposit Account No. **19-1135** in the amount of **\$162.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **19-1135** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Daniel A. Monaco
SEIDEL GONDA LAVORGNA & MONACO, P.C.
 Two Penn Center, Suite 1800
 Philadelphia, PA 19102
 (215) 568-8383

Daniel A. Monaco
 SIGNATURE
DANIEL A. MONACO
 NAME
30,480
 REGISTRATION NUMBER
JAN-20, 2001
 DATE

09/744406

J007 Rec'd PCT/PTC 22 JAN 2001

ALLOGENEIC CELLULAR IMMUNOGENS USEFUL AS CANCERVACCINESCross-Reference to Related Application

5 This application claims the benefit of United States Provisional
Application 60/093,965 filed July 24, 1998.

Field of the Invention

The invention relates to the field of cancer vaccination and immunotherapy.

Background of the Invention

10 A current goal of cancer research is the identification of host
factors that either predispose to tumor formation or serve to enhance tumor
growth.

Genes that confer the ability to convert cells to a tumorigenic state
are known as **oncogenes**. The transforming ability of a number of retroviruses
15 has been localized in individual viral oncogenes (generally *v-onc*). Cellular
oncogenes (generally *c-onc*) present in many species are related to viral
oncogenes. It is generally believed that retroviral oncogenes may represent
escaped and/or partially metamorphosed cellular genes that are incorporated into
the genomes of transmissible, infectious agents, the retroviruses.

20 Some *c-onc* genes intrinsically lack oncogenic properties, but may
be converted by mutation into oncogenes whose transforming activity reflects the

acquisition of new properties, or loss of old properties. Amino acid substitution can convert a cellular proto-oncogene into an oncogene. For example, each of the members of the *c-ras* proto-oncogene family (*H-ras*, *N-ras* and *K-ras*) can give rise to a transforming oncogene by a single base mutation.

5 Other *c-onc* genes may be functionally indistinguishable from the corresponding *v-onc*, but are oncogenic because they are expressed in much greater amounts or in inappropriate cell types. These oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized example of this type of proto-oncogene is *c-myc*.
10 *MYC* protein sequence does not appear to be essential for oncogenicity. Overexpression or altered regulation is responsible for the oncogenic phenotype. Activation of *c-myc* appears to stem from insertion of a retroviral genome within or near the *c-myc* gene, or translocation to a new environment. A common feature in the translocated loci is an increase in the
15 level of *c-myc* expression.

Gene amplification provides another mechanism by which oncogene expression may be increased. Many tumor cell lines have visible regions of chromosomal amplification. For example, a 20-fold *c-myc* amplification has been observed in certain human leukemia and lung carcinoma
20 lines. The related oncogene *N-myc* is five to one thousand fold amplified in human neuroblastoma and retinoblastoma. In human acute myeloid leukemia and colon carcinoma lines, the proto-oncogene *c-myb* is amplified five to ten fold. While established cell lines are prone to amplify genes, the presence of known oncogenes in the amplified regions, and the consistent amplification of particular
25 oncogenes in many independent tumors of the same type, strengthens the correlation between increased expression and tumor growth.

Immunity has been successfully induced against tumor formation by inoculation with DNA constructs containing *v-onc* genes, or by inoculation with *v-onc* proteins or peptides. A series of reports describe a form of
30 "homologous" challenge in which an animal test subject is inoculated with either

5 v-*src* oncoprotein or DNA constructs containing the v-*src* gene. Protective immunity was induced against tumor formation by subsequent challenge with v-*src* DNA or v-*src*-induced tumor cells. See, Kuzumaki *et al.*, *JNCI* (1988), 80:959-962; Wisner *et al.*, *J. Virol.* (1991), 65:7020-7024; Halpern *et al.*, *Virology* (1993), 197:480-484; Taylor *et al.*, *Virology* (1994), 205:569-573; Plachy *et al.*, *Immunogenetics* (1994), 40:257-265. A challenge is said to be "homologous" where reactivity to the product of a targeted gene is induced by immunization with the same gene, the corresponding gene product thereof, or fragment of the gene product. A challenge is "heterologous" where reactivity to the product of a targeted gene is induced by immunization with a different gene, gene product or fragment thereof.

10 WO 92/14756 (1992) describes synthetic peptides and oncoprotein fragments which are capable of eliciting T cellular immunity, for use in cancer vaccines. The peptides and fragments have a point mutation or translocation as compared to the corresponding fragment of the proto-oncogene. The aim is to induce immunoreactivity against the mutated proto-oncogene, not the wild-type proto-oncogene. WO 92/14756 thus relates to a form of homologous challenge.

15 EP 119,702 (1984) describes synthetic peptides having an amino acid sequence corresponding to a determinant of an oncoprotein encoded by an oncoviral virus, which determinant is vicinal to an active site of the oncoprotein. The active site is a region of the oncoprotein required for oncoprotein function, e.g., catalysis of phosphorylation. The peptides may be used to immunize hosts to elicit antibodies to the oncoprotein active site. EP 119,702 is thus directed to a form of homologous challenge.

25 The protein product encoded by a proto-oncogene constitutes a self antigen and, depending on the pattern of its endogenous expression, would be tolerogenic at the level of T cell recognition of the self peptides of this product. Thus, vaccination against cancers which derive from proto-oncogene overexpression is problematic.

Recent attempts have been made to induce immunity *in vitro* or *in vivo* to the product of the HER-2/*neu* proto-oncogene. The proto-oncogene encodes a 185-kDa transmembrane protein. The HER-2/*neu* proto-oncogene is overexpressed in certain cancers, most notably breast cancer. In each report discussed below, the immunogen selected to induce immunity comprised a purified peptide of the p185^{HER-2/*neu*} protein, and not a cellular immunogen.

Disis *et al.*, *Cancer Res.* (1994) 54:16-20 identified several breast cancer patients with antibody immunity and CD4+ helper/inducer T-cell immunity responses to p185^{HER-2/*neu*} protein. Antibodies to p185^{HER-2/*neu*} were identified in eleven of twenty premenopausal breast cancer patients. It was assumed prior to this work that patients would be immunologically tolerant to HER-2/*neu* as a self-protein and that immunity would be difficult to generate.

Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076 constructed synthetic peptides identical to p185^{HER-2/*neu*} protein segments with amino acid motifs similar to the published motif for HLA-A2.1-binding peptides. Out of four peptides synthesized, two were shown to elicit peptide-specific cytotoxic T-lymphocytes by primary *in vitro* immunization in a culture system using peripheral blood lymphocytes from a normal individual homozygous for HLA-A2. Thus, it was concluded that the p185^{HER-2/*neu*} proto-oncogene protein contains immunogenic epitopes capable of generating human CD8⁺ cytotoxic T-lymphocytes.

The cytotoxic T cells elicited in the latter report were not, however, shown to recognize tumor cells, but only targets that bound the synthesized peptides. Other work (Dahl *et al.*, *J. Immunol.* (1996), 157:239-246) has demonstrated that cytotoxic cells may recognize targets that bind peptide but fail to recognize targets that endogenously synthesize peptide. It is thus unclear whether the cytotoxic cells elicited by Disis *et al.* would be capable of recognizing tumor cells. In any event, no protection against tumor growth was demonstrated by Disis *et al.*

Peoples *et al.*, *Proc. Natl. Acad. Sci. USA* (1995), 92:432-436, report the identification of antigenic peptides presented on the surface of ovarian and breast cancer cells by HLA class I molecules and recognized by tumor-specific cytotoxic T lymphocytes. Both HLA-A2-restricted breast and ovarian tumor-specific cytotoxic T lymphocytes recognized shared antigenic peptides. T cells sensitized against a nine-amino acid sequence of one of the peptides demonstrated significant recognition of HLA-A2 HER2/*neu* tumors.

It remains unclear whether Peoples *et al.* have successfully attacked proto-oncogene-encoded self, as the immunizing peptide which is expressed in the tumor cells contained an isoleucine at position 2, whereas the peptide expressed in normal tissue contains valine residue at this position. Moreover, although stimulation of T cells occurred *in vitro*, this stimulation does not represent a true primary immune response insofar as the starting T cell population represented tumor infiltrating lymphocytes.

The research accounts of Disis *et al.* and Peoples *et al.* required a form of *in vitro* stimulation, either priming as described by Disis *et al.* or restimulation as described by Peoples *et al.* The *in vitro* protocols of Disis *et al.* and Peoples *et al.* require a mutant cell line to aid in selection of the peptide which will serve to induce reactivity. Non-mutant, peptide antigen-presenting cells have their HLA class I molecules already loaded with endogenous peptides, a phenomenon which precludes exogenous loading from without. The value of the mutant lines is that they lack the TAP genes (encoding the transporters associated with antigen presentation). Class I binding of internally-derived peptides is significantly lowered, and "empty" class I molecules are present on the cell surface and available for binding of exogenously added peptides. This availability of peptide binding sites on membrane-bound class I allows examination of whether a given peptide will (i) even bind to class I, and (ii) function as a target in cytotoxic T cell assays. However, the need for a mutant cell line for deduction of candidate immunizing peptide sequences limits the usefulness of peptide-based immunization schemes.

Fendly *et al.*, *J. Biol. Response Modifiers* (1990), 9:449-455 present an account of a polypeptide-based immunotherapy. Purified polypeptide corresponding to the extracellular domain of the p185^{HER-2/neu} protein was obtained from a transfected cell line. The purified peptide was employed in the immunization of guinea pigs. The immunized animals developed a cellular immune response, as monitored by delayed-type hypersensitivity. Antisera derived from immunized animals specifically inhibited the *in vitro* growth of human breast tumor cells overexpressing p185^{HER-2/neu}. There is no indication by Fendly *et al.* of induction of self versus non-self reactivity. It is likely that the guinea pigs were chiefly responding to non-self determinants (as defined in terms of the guinea pig host) on the human polypeptide immunogen.

The use of peptides for immunization is of necessity limited to immunization with a single haplotype. There are approximately thirty HLA types in man. In each case of peptide immunization, one must be careful to select peptides which match the host HLA type. The selected peptide must be immunogenic in the host and be capable of presentation to host immune system cells.

What is needed is an immunization method for immunizing humans and animals against self-encoded proto-oncogenes which are associated with the development of cancer, which dispenses with the need for isolating immunogenic, HLA host-matched peptides for immunization.

09744406.012201

Summary of the Invention

It is an object of the invention to induce reactivity to self-determinants of the product of an overexpressed proto-oncogene.

5 It is an object of the invention to provide for a form of therapy or prophylaxis based upon the capacity to induce immune reactivity to proto-oncogene-encoded self as overexpressed in tumor cells.

It is an object of the invention to provide a cellular immunogen for use in immunization against self proto-oncogene determinants.

10 It is an object of the invention to provide for a method for vaccinating a host against disease associated with the overexpression of a proto-oncogene.

These and other objects will be apparent from the following disclosure.

15 A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene is provided. The method comprises:

(a) transfecting allogeneic donor cells with
at least one transgene construct comprising at least
one transgene cognate to the target proto-oncogene
and a strong promoter to drive the expression of
the transgene in the transfected cells, the transgene
encoding a gene product which induces host
immunoreactivity to host self-determinants of the
product of the target proto-oncogene gene;

20 (b) inserting the cells transfected with the
transgene construct into the body of the host to
obtain expression of the transgene in the host.

25 According to one principal embodiment of the invention, the transgene comprises wild-type or mutant retroviral oncogene DNA. According to another principal embodiment of the invention, the transgene comprises wild-
30 type or mutant proto-oncogene DNA of a species different from the host species.

09744406 012201

Where the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA, the mutant DNA is preferably nontransforming. The mutant DNA preferably comprises a deletion mutation in a region of the DNA which is essential for transformation. Preferably, the donor cells are transfected with a plurality, most preferably at least five, different transgene constructs, each construct encoding a different deletion mutation.

In one preferred embodiment of the invention, the mutant DNA has at least about 75% homology, more preferably at least about 80% homology, most preferably at least about 90% homology, with the corresponding wild-type oncogene or proto-oncogene DNA.

The invention is further directed to a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which is associated with a cancer. The cellular immunogen comprises the allogeneic donor cells which have been transfected with at least one transgene construct, as described above.

The invention is also directed to a method of preparing the cellular immunogen, by (a) providing allogeneic donor cells, and (b) transfecting the cells with at least one transgene construct, as described above.

The cells transfected with the transgene are preferably rendered non-dividing prior to insertion into the body of the host.

The term "allogeneic" as used herein to describe the genetic relationship between organisms has its ordinary meaning in biology, that is, organisms are allogeneic if they are genetically dissimilar (i.e., non-identical) members of the same species. Cells comprising an "allogeneic" graft are derived from organisms which are the same species as the host, but are not genetically identical.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human *c-myc* gene is the cognate gene to the mouse *c-myc* gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode proteins which are functionally equivalent.

By "donor cell" is meant a cell which is used herein for immunization of a host. The expression "donor cell" is meant to include not only cells freshly harvested from donor individuals, but also cultured cells which may have been obtained from a cell or tissue bank or other biological repository. By "allogeneic donor cell" is meant a cell which is allogeneic with respect to the host, i.e., the donor cell, upon transplantation into the host, would compromise an allogeneic graft.

By "homology" is meant the degree of sequence similarity between two different amino acid sequences, as that degree of sequence similarity is derived by the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

The word "transfection" is meant to have its ordinary meaning, that is, the introduction of foreign DNA into eukaryotic cells.

By "transgene" is meant a foreign gene that is introduced into one or more donor cells.

By "transgene construct" is meant DNA containing a transgene and additional regulatory DNA, such as promoter elements, necessary for the expression of the transgene in the donor cells used to inoculate the host.

Description of the Figures

5 Figs. 1A and 1B are plots of the mean tumor diameter over time following subcutaneous wing inoculation of 1-day-old line TK (Fig. 1A) and line SC (Fig. 1B) chickens with 100 μ g of tumorigenic plasmids *pcsrc527* (—▲—), *pVSRC-C1* (—●—) or *pMVsrc* (—■—). The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated
10 was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for *pcsrc527*, italics for *pVSRC-C1*, bold typeface for *pMVsrc*). Error bars (unless obscured by the
15 symbol) indicate standard error.

Figs. 2A and 2B are plots of the growth of challenge (wing web) tumors in test and control line TK chickens under conditions of (i) priming and homologous challenge with plasmid *pcsrc527* (Fig. 2A: --△--, test; --▲--, control), or (ii) priming and homologous challenge with plasmid *pVSRC-C1*
20 (Fig. 2B: --○--, test; --●--, control). Test chickens were primed at 1 day posthatch with 100 μ g of construct; test and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge diameter was computed as in Figs. 1A and 1B. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is
25 indicated (standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's *t* test, *(*p* < 0.05). **(*p* < 0.01). ***(*p* < 0.001). The statistical comparison between the ratios of chickens bearing palpable challenge tumors to

total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where $p < 0.05$. Error bars indicate standard error.

Figs. 3A and 3B are plots of the growth of challenge (wing web) tumors in TK chickens under conditions of (i) priming with plasmid pVSRC-C1 and heterologous challenge with plasmid pcsrc527 (Fig. 3 A: --△--, test; --▲--, control) or (ii) priming with pcsrc527 and heterologous challenge with pVSRC-C1 (Fig. 3B: --○--, test; --●--, control). Test chickens were primed at 1 day posthatch with 100 μ g of construct; test and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge tumor diameter was computed as in Figs. 1A and 1B. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described for Figs. 2A and 2B. [$*(p < 0.05)$, $**(p < 0.01)$, $*** (p < 0.001)$, for the student's *t* test], and the paired ratios are underlined for only those time points where, in the chi-squared test, $p < 0.05$. Error bars indicate standard error.

Detailed Description of the Invention

A vaccination strategy is provided to prevent development of cancers. The vaccination method may be carried out on a subject at risk for a particular cancer, but before the development of the cancer. The practice of the invention may serve for the immunoprevention of prevalent human cancers, such as colon carcinoma, breast carcinoma, and various lymphomas whose progress is accompanied by the overexpression of a cellular proto-oncogene.

The vaccination strategy of the present invention relies on the induction of an immune response that targets tumor cells by virtue of the recognition of the proto-oncogene-specific antigenicity. The aim of the vaccine protocol is to induce reactivity to self-determinants of an overexpressed proto-

oncogene product. The strategy exploits the structural relatedness between the product of the cellular proto-oncogene and that of the product of genes cognate to the target proto-oncogene. The cognate gene may comprise a wild-type or mutant cognate retroviral oncogene or a wild-type or mutant proto-oncogene of a species different from the host species. The starting point of the vaccine strategy is the high degree of primary sequence homology that exists between the protein product of a targeted proto-oncogene and that of its cognate retroviral oncogene, or between the proto-oncogene product and the product of a cognate proto-oncogene from a different species. However, in contrast to other proposed vaccine strategies, the present invention is not based on the immune recognition of a determinant defined by a cancer specific mutation.

For those tumors showing proto-oncogene overexpression, this sequence homology permits application of the following strategy, which can be employed either prophylactically or therapeutically under conditions of cell-surface expression, or other forms of adjuvancity, as chosen to enhance immunogenicity: (a) transfection of allogeneic donor cells with a DNA construct comprising a transgene cognate to the target proto-oncogene, which transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene; (b) transplantation of the transfected cells into the body of the host to obtain expression of the transgene in the host, and thus immunity against the proto-oncogene product. The invention relies on the targeting of a self-determinant found on an overexpressed or overabundant proto-oncogene-encoded product. The foreign peptide elements of the immunizing oncogene product will trigger peripheral lymphocytes exhibiting a weak cross reactivity for the self peptides of the targeted proto-oncogene product. Although such self peptides would be present in normal cells expressing the proto-oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

The immune strategy exploits the antigenicity of two alternative types of determinants: (1) tumor-associated antigenic determinant(s) induced as

09744406.0122001
102210.9044460

a consequence of the activity of the oncogene product, e.g., an enzymatic modification of a cellular protein effected by the oncogene product, or (2) tumor associated antigenic determinant(s) intrinsic to the oncogene-encoded product itself. The difficulty in exploiting the first alternative by traditional means, i.e., antigen purification, is that at present little or no systematic information exists bearing on the properties of an antigen that, though oncogene-induced, is not oncogene-encoded. This situation makes purification of any such antigen problematic. However, this problem is obviated from the outset by the present invention which utilizes allogeneic donor cells which, as transfected in culture by the cognate retroviral oncogene, would express the relevant antigenicity.

In terms of exploiting the second alternative, that of an antigenicity intrinsic to the proto-oncogene product, a relevant consideration is that the protocol of immunization according to the present invention primes the host to determinants of the oncogene product itself. A consequence of this immunization is induction of T-cell reactivity to the divergent, i.e. foreign, peptide determinants of the retroviral oncogene product, i.e., those peptide determinants that show sequence differences with the positionally homologous determinants of the cellular proto-oncogene product. The induction of this reactivity does not in itself have vaccine potential, since the foreign determinants specific to the retroviral oncogene product are normally absent from the cellular proto-oncogene product. Nevertheless, the foreign peptide elements, notably those that differ by only a single amino acid from the positionally homologous self peptides, trigger peripheral T-lymphocytes exhibiting a weak cross-reactivity for the self peptides. Although such self peptides are present in normal cells expressing the proto-oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

It is possible that many tumor-associated and overexpressed proto-oncogenes might possess mutations. In some cases, overexpression may very well arise as a direct consequence of one or more of the mutations. However, the present vaccination method does not have as its object the deliberate targeting of

non-self determinants generated by proto-oncogene mutations. Unlike prior vaccination methods designed to target such mutation-driven non-self determinants, it is the aim of the present invention to induce reactivity for self-determinants in the overexpressed product of tumor associated and overexpressed proto-oncogenes.

Prior efforts attempting to elicit reactivity to proto-oncogene self determinants have relied on *in vitro* protocols utilizing mutant cell lines to identify individual self peptide immunogens (Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076; Peoples *et al.*, *Proc. Natl. Acad. Sci USA* (1995), 92:432-436).

According to the present invention, the host immune system is presented with the full array of naturally-derived class I binding peptides. The vaccine strategy of the present invention obviates the need for any *a priori* assessment of the immunogenicity of individual peptides.

While the cellular immunogens of the invention display self peptides, non-self peptides would also be presented which may serve as more effective tolerance breakers. The value of a non-self, but closely related to self, peptide is that it may more readily activate those T cells that have both a weak cross reactivity for the cognate self peptide and an activation threshold (determined by the tightness of binding to the T cell receptor) too high to be triggered by the self peptide. Moreover, cognate non-self is inductive of a good immune response, simply because it does in fact constitute nonself. The non-self immune response is expected to predispose the induction of the inevitably weaker response to the self determinants on the same protein product, since the resultant cytokine release provides local help to initiate the weaker anti-self response.

As hereinafter exemplified in a model of *src*-oncogene-based tumor formation, immunization with cells transfected with a transgene construct expressing the v-*src* oncogene product induces reactivity to the product of the c-*src* proto-oncogene, thereby conferring protection against the growth of tumors displaying overexpression of the c-*src* proto-oncogene.

We have described a similar vaccination strategy in PCT/US97/00582. However, the methodology described therein utilizes excised host cells for preparing the cellular immunogen. According to the present invention, the cellular immunogen is prepared from cells obtained from a donor other than the patient, and other than an identical twin of the patient. Hence, while PCT/US97/00582 describes an inoculation constituting a syngeneic transfer, the present invention relies upon an allogeneic transfer. Given the outbredness of the human population, there will inevitably be allelic differences between the donor and the patient. These differences do not impede the induction of immunity to the cognate proto-oncogene encoded product overexpressed in the allogeneic transfectants used as immunogen.

Until recently, it was believed that antigen presentation was restricted to those class I alleles of the major histocompatibility complex (MHC) that were shared between antigen presenting cells and T cells, a concept that would imply that fibroblasts mismatched at a HLA (human leukocyte antigen) class I allele of the human MHC could not present antigen in the context of this allele. It is now known that this view is too narrow. In fact, antigen released from transplanted donor cells or cell debris will be internalized by a recipient's bone marrow-derived antigen presenting cells, then processed by these cells for presentation in the context of the class I alleles of the recipient. See Huang *et al.*, *Science* 264:961-964 (1994).

This pathway of antigen transfer from the donor cells to the bone marrow-derived antigen presenting cells of the recipient exemplifies an *in vivo* process designated the "exogenous pathway", to distinguish it from the "classical pathway"; the latter as based on processing of antigen-presenting cells themselves.

The operation of the exogenous pathway permits recipient priming to a cellular antigen introduced in the context of a non-recipient class I allele, thereby permitting the use of allogeneic donor cells, mismatched with reference to the recipient at none or at all of the diploid 6 human class I loci, for priming to an overexpressed cognate proto-oncogene.

Target Proto-Oncogenes

According to the present invention, patients with a family history of a cancer characterized by the overexpression of a particular proto-oncogene are selected for immunization. Alternatively, patients whose tumors can be shown to overexpress the proto-oncogene are selected. Overexpression of a proto-oncogene may derive from an increase over a basal level of transcription. Overexpression may also derive from gene amplification, that is, an increase in gene copy number, coupled with a basal or elevated level of transcription. Proto-oncogene overexpression may be assayed by conventional probing techniques, such as described in *Molecular Cloning: A Laboratory Manual* J. Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1989. The level of target proto-oncogene expression may be determined by probing total cellular RNA from patient cells with a complementary probe for the relevant mRNA. Total RNA from the patient cells is fractionated in a glyoxal/agarose gel, transferred to nylon and hybridized to an appropriately labelled nucleic acid probe for the target mRNA. The number of relevant mRNA transcripts found in the patient cells is compared to that found in cells taken from the same tissue of a normal control subject.

As an alternative to measuring mRNA transcripts, the expression level of a target proto-oncogene may be assessed by assaying the amount of encoded protein which is formed. Western blotting is a standard protocol in routine use for the determination of protein levels. See *Molecular Cloning, supra*. Chapter 18, incorporated herein by reference. Accordingly, a cell lysate or other cell fraction containing protein is electrophoresed on a polyacrylamide gel, followed by protein transfer to nitrocellulose, and probing of the gel with an antibody specific for the protein in question. The probe step permits resolution of the desired protein from all other proteins in the starting mixture. The bound antibody may be prelabelled, *e.g.*, by a radioisotope such as ^{125}I , so as to permit its detection on the gel. Alternatively, a secondary reagent (usually an anti-

immunoglobulin or protein A) may be radiolabeled or covalently coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase. The strength of the signal is proportional to the amount of the target protein. The strength of the signal is compared with the signal from a sample analyzed in the same manner.

5 but taken from normal as opposed to tumor tissue.

A description of the methodology and use of Western blotting to determine the levels of the c-src-encoded protein pp60^{c-src} in adenomatous polyps (colonic epithelia) is provided by Cartwright *et al.*, *Proc. Natl. Acad. Sci. USA* (1990), 87:558-562, the entire disclosure of which is incorporated herein by
10 reference.

An at least about eight-fold increase in that gene's expression in the patient cells compared to expression in normal control cells from the same tissue would indicate candidacy for vaccination.

Table 1 includes a partial list of representative proto-oncogenes, the overexpression of which has been associated with one or more malignancies. Each listed proto-oncogene is a target proto-oncogene according to the present invention. The corresponding oncogene, of which the target proto-oncogene is the normal cellular homolog, is also identified. This list of target proto-oncogenes is intended to be representative, and not a complete list.

20 **Table 1**

Representative List of Target Proto-Oncogenes

| <u>Proto-Oncogene</u> | <u>Tumor</u> | <u>Comments/References</u> |
|-----------------------|--------------|--|
| AKT-2 | ovarian | v-Akt is the oncogene of the AKT8 virus, which induces lymphomas in mice. 1. Bellacosa <i>et al.</i> , (1995) <i>Int. J. Cancer</i> 64(4):280-5: Southern-blot analysis has shown AKT-2 amplification in 12.1% of ovarian |

09744406-012201

carcinomas, while Northern blot analysis has revealed overexpression of AKT-2 in 3 of 25 fresh ovarian carcinomas which were negative for AKT-2 amplification.

- 5 2. Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 89(19): 9267-71: Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.

10 AKT-2 pancreatic Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93(8):3636-41: Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.

15 c-erbB-2 bladder c-ErbB-2 is also known as HER2/neu. V-erbB is the oncogene of the avian erythroblastosis virus.
1. Underwood *et al.*, (1995) *Cancer Res.* 55(11):2422-30: Protein overexpression was observed in 45% of patients with non-recurrent disease and 50% of patients with recurrent disease; 9% of bladder tumors analyzed showed gene amplification.

20 2. Coombs *et al.*, (1993) *Pathology* 169(1):35-42: c-ErbB-2 gene amplification was observed in 14% of bladder tumors analyzed.

25 3. Gardiner *et al.*, (1992) *Urolog. Res.* 20(2):17-20: Nineteen percent of primary transitional cell bladder carcinomas showed c-erbB-2 gene amplification.

c-erbB-2 breast

1. Molina *et al.*, (1966) *Anticancer Research* 16(4B):2295-300: Abnormal c-erbB-2 levels were found in 9.2% of patients with locoregional breast carcinoma, and in 45.4% of patients with advanced disease.
2. DePotter *et al.*, (1995) *Virchows Arch.* 426(2):107-15: Overexpression of the oncoprotein is observed in about 20% of invasive duct cell carcinomas of the breast.
3. Bandyopadhyay *et al.*, (1994) *Acta Oncol.* 33(5):493-8: 35.4% of breast tumors showed c-erbB-2 overexpression; 17.4% showed gene amplification.
4. Fontana *et al.*, (1994) *Anticancer Res.* 14(5B):2099-104: 26% of samples showed c-erbB-2 amplification.
5. Press *et al.*, (1993) *Cancer Research* 53(20):4960-70: Amplified overexpression was identified in 38% of primary breast cancers.
6. Berns *et al.*, (1992) *Cancer Res.* 52(5):1107-13: 23% of primary breast cancer tissues exhibited amplification.
7. Delvenne *et al.*, (1992) *Eur. J. of Cancer* 28(2-3):700-5: c-erbB-2 mRNA was overexpressed in 34% of breast tumor samples.
8. Inglehart, (1990) *Cancer Res.* 50(20):6701-7: Two to thirty-two-fold gene amplification was found in multiple stages of tumor progression.
9. Slamon *et al.*, (1989) *Science* 244:707-12: A 28% incidence of amplification of c-erbB-2 was found in 189 primary breast cancers.
10. Kraus *et al.*, (1987) *EMBO J.* 6(3):605-10: Eight cell lines demonstrated c-erbB-2 mRNA levels ranging from

4 to 128-fold overexpression. 60% of all tumors analyzed showed elevated levels of *c-erbB-2* mRNA.

- | | | | |
|----|-----------------|---------|--|
| | <i>c-erbB-2</i> | lung | 1. Osaki <i>et al.</i> , (1995) <i>Chest</i> 108(1):157-62: |
| 5 | | | Lung tissue overexpression of <i>c-erbB-2</i> was |
| | | | discovered in 42.5% of samples. 2. Lorenz <i>et al.</i> , |
| | | | (1994) <i>Clin. Invest.</i> 72(2):156-63: A 64-fold |
| | | | increase in the amount of <i>c-erbB-2</i> mRNA was |
| 10 | | | observed; 33% of lung tumors showed |
| | | | overexpression of <i>c-erbB-2</i> . |
| | <i>c-erbB-2</i> | ovarian | 1. Katsaros <i>et al.</i> , (1995) <i>Anticancer Res.</i> |
| | | | 15(4):1501-10: Abnormally high expression of <i>c-</i> |
| | | | <i>erbB-2</i> was found in 31% of tumor samples. 2. |
| 15 | | | Felip <i>et al.</i> , (1995) <i>Cancer</i> 75(8):2147-52: 21.7% |
| | | | of ovarian tumors showed overexpression of <i>c-</i> |
| | | | <i>erbB-2</i> . 3. Fan <i>et al.</i> , (1994) <i>Chin. Med. J.</i> |
| | | | 107(8):589-93: <i>c-erbB-2</i> amplification was found |
| | | | in 30.8% (8 of 26) of human ovarian cancers. 4. |
| 20 | | | vanDam <i>et al.</i> , (1994) <i>J. of Clin. Path.</i> |
| | | | 47(10):914-9: 24% of ovarian tumors showed <i>c-</i> |
| | | | <i>erbB-2</i> overexpression. 5. Csokay <i>et al.</i> , (1993) |
| | | | <i>Eur. J. of Surg. Oncology</i> 19(6):593-9: <i>c-erbB-2</i> |
| | | | amplification was found in 34% of fresh ovarian |
| 25 | | | tumor samples. 6. McKenzie <i>et al.</i> , (1993) |
| | | | <i>Cancer</i> 71(12):3942-5: 30% of ovarian tumor |
| | | | samples indicated <i>c-erbB-2</i> overexpression. 7. |
| | | | Hung <i>et al.</i> , (1992) <i>Cancer Letters</i> 61(2):95-103: |
| | | | A 100-fold <i>c-erbB-2</i> overexpression was |

discovered in one human cell line. Two to four-fold amplification was also discovered.

- | | | | |
|----|--------------|----------|--|
| | <i>MDM-2</i> | leukemia | <i>MDM-2</i> is the murine double minute-2 oncogene. |
| 5 | | | 1. Bueso-Ramos <i>et al.</i> , (1993) <i>Blood</i> 82(9):2617-23: 53% of cases showed overexpression of <i>MDM-2</i> mRNA. The level of <i>MDM-2</i> mRNA overexpression in some cases of leukemias was comparable to that observed in some sarcomas, which demonstrate more than 50-fold <i>MDM-2</i> gene amplification. No evidence of gene amplification was observed. |
| 10 | | | 2. Watanabe <i>et al.</i> , (1994) <i>Blood</i> 84(9):3158-65: 28% of patients with B-cell chronic lymphocytic leukemia or non-Hodgkin's lymphoma had 10-fold higher levels of <i>MDM-2</i> gene expression. <i>MDM-2</i> overexpression was found more frequently in patients at advanced clinical stages. |
| 15 | | | |
| | <i>c-myb</i> | colon | <i>V-myb</i> is the oncogene of the avian myeloblastoma virus. 1. Ramsay <i>et al.</i> , (1992) <i>Cell Growth and Diff.</i> 3(10):723-30: <i>c-myb</i> levels were always higher in colon cancer samples than normal tissue. |
| 20 | | | 2. Alitalo <i>et al.</i> , (1984) <i>Proc. Natl. Acad. Sci.</i> 81(14):4534-8: <i>c-myb</i> levels were always higher in colon cancer samples than normal tissue. |
| 25 | <i>c-myc</i> | breast | <i>V-myc</i> is the oncogene of the avian myelocytoma virus. 1. Lonn <i>et al.</i> , (1995) <i>Cancer</i> 75(11):2681-7: Amplification of <i>c-myc</i> occurs in 16% of |

- patients with breast cancer. 2. Hehir *et al.*, (1993) *J. of Surg. Oncology* 54(4):207-9: *c-myc* overexpression was found in 60% of breast carcinoma samples. 3. Kreipe *et al.*, (1993) *Cancer Research* 53(8):1956-61: Amplification of *c-myc* was found in 52.6% of samples that displayed a Ki-S1 labelling index exceeding 30%.
4. Watson *et al.*, (1993) *J. Nat. Cancer Inst.* 85(11):902-7: Amplification of *c-myc* occurs in up to 20 - 30% of breast cancers. 5. Berns *et al.*, (1992) *Cancer Research* 52(5):1107-13: Amplification was found in 20% of primary breast cancer patients: the range was 3-14 gene copies.
6. Watanabe *et al.*, (1992) *Cancer Research* 52(19):5178-82: Expression of *c-myc* was increased by 10-fold.
- c-myc* gastric/
 colorectal
1. Rigas, (1990) *Clin. Gastroent.* 12(5):494-9: Overexpression of *c-myc* is found in 80 of colon cancers. 2. Erisman *et al.*, (1988) *Oncogene* 2(4):367-78: Adenocarcinoma cell lines express 5-10-fold elevated levels of *c-myc* mRNA. Eight to thirty-seven-fold higher levels of *c-myc* protein was found in tumor cell lines compared to normal cells.
3. Sikora *et al.*, (1987) *Cancer* 59(7):1289-95: Up to 32-fold overexpression of *c-myc* mRNA was observed in 12 to 15 tumors. 4. Tsuboi *et al.*, (1987) *Biochem. and Biophys. Res. Comm.* 146(2):705-10: Gastric Cancer: A 2-3-fold overexpression was observed in gastric cancer. A

2-10-fold overexpression was observed in colorectal cancer.

- | | | | |
|----|-------|-----------------|--|
| | c-myc | lung | 1. Lorenz <i>et al.</i> , (1994) <i>Clin. Invest.</i> 72(2):156-63: A 57-fold increase in c-myc mRNA levels was observed. 23% of samples indicated strong expression of c-myc. 2. Kato <i>et al.</i> , (1993) <i>Jap. J. of Cancer Res.</i> 84(4):355-9: Liver tissue metastases from human small cell lung carcinoma revealed 30-fold amplification of c-myc. |
| 5 | | | |
| 10 | c-myc | naso-pharyngeal | Porter <i>et al.</i> , (1994) <i>Acta Oto-Laryng.</i> 114(1):1105-9: 22% of samples showed intense staining for c-myc. |
| | c-myc | ovarian | 1. Bian <i>et al.</i> , (1995) <i>Chin. J. of Ob. Gyn.</i> 30(7):406-9: 50% of samples showed amplification of c-myc. 2. Katsaros <i>et al.</i> , (1995) <i>Anticancer Res.</i> 15(4):1501-10: 26% of samples exhibited c-myc amplification. 3. van Dam <i>et al.</i> , (1994) <i>J. Clin. Path.</i> 47(10):914-9: Overexpression of c-myc was found in 35% of ovarian carcinomas. 4. Xin <i>et al.</i> , (1993) <i>Chin. J. of Ob. Gyn.</i> 28(7):405-7: 54.5% of samples showed amplification of c-myc. 5. Tashiro <i>et al.</i> , (1992) <i>Int. J. of Cancer</i> 50(5):828-33: Overexpression was found in 63.5% of all serous adenocarcinoma tissues and 37.3% of all ovarian carcinoma tissues. Significant overexpression of c- |
| 15 | | | |
| 20 | | | |
| 25 | | | |

myc was observed at Stage III compared with other stages.

- | | | | |
|----|-------|----------|--|
| 5 | c-myc | prostate | Nag <i>et al.</i> , (1989) <i>Prostate</i> 15(2):115-22: A 10-fold amplification of c-myc was observed. Fifty-fold higher levels of mRNA transcripts of c-myc were found. |
| 10 | c-ras | lung | <i>Ras</i> oncogenes were first recognized as the transforming genes of Harvey and Kirsten murine sarcoma viruses. Lorenz <i>et al.</i> , (1994) <i>Clin. Invest.</i> 72(2):156-63: a 13-fold increase in overexpression of c-Ki-ras was observed. 18% of tumors displayed strong overexpression of c-Ki-ras. |
| 15 | c-ras | ovarian | 1. Katsaros <i>et al.</i> , (1995) <i>Anticancer Res.</i> 15(4):1501-10: Higher levels of <i>ras</i> protein than in normal or benign ovarian tumors were found in 45% of tumor samples. 2. vanDam <i>et al.</i> , (1994) <i>J. of Clin. Path.</i> 47(10):914-9: 20% of ovarian tumors exhibited c-ras overexpression. The levels of expression of c-ras were much higher in tumors of patients with recurrent or persistent disease after chemotherapy, than in the tumors of patients at initial presentation. |
| 20 | | | |
| 25 | c-src | breast | V-src is the oncogene of the Rous sarcoma virus, which induces sarcomas in chickens. Muthuswamy <i>et al.</i> , (1994) <i>Mol. and Cell. Biol</i> |

14(1):735-43: *c-erbB-2*-induced mammary tumors possessed 6-8-fold higher *c-src* kinase activity than adjacent epithelium.

- | | | | |
|----|--------------|------------|--|
| | <i>c-src</i> | colon/ | 1. Cartwright <i>et al.</i> , (1994) <i>J. of Clin. Invest.</i> |
| 5 | | colorectal | 93(2):509-15: <i>c-src</i> activity is 6-10-fold higher in mildly dysplastic ulcerative colitis (a chronic inflammatory disease of the colon with a high on incidence of colon cancer) than in non-dysplastic epithelia. This data suggests that activation of <i>c-src</i> is an early event in the genesis of UC colon cancer. |
| 10 | | | 2. Talamonti <i>et al.</i> , (1993) <i>J. of Clin. Invest.</i> 91(1):53-60: High level of <i>c-src</i> activity from colorectal cancer is found in liver metastases. |
| 15 | | | 3. Termuhlen <i>et al.</i> , (1993) <i>J. of Surg. Res.</i> 54(4):293-8: Colon carcinoma metastases to the liver had significantly increased activity of <i>c-src</i> with an average 2.2-fold increase. Extrahepatic colorectal metastases demonstrated an average 12.7-fold increase in <i>c-src</i> activity over normal mucosa. |
| 20 | | | |
| | <i>c-yes</i> | colon | <i>V-yes</i> is the oncogene of two avian sarcoma viruses, Esh sarcoma virus and Y73. 1. Pena <i>et al.</i> , (1995) <i>Gastroent.</i> 108(1):117-24: Twelve to fourteen-fold higher expression of <i>c-yes</i> was found in colonic transforming oncogene adenomas compared to normal mucosa. Activity of <i>c-yes</i> was elevated in adenomas that are at greatest risk for developing cancer. 2. Park <i>et al.</i> , (1993) |
| 25 | | | |

Oncogene 8(10):2627-35: A ten to 20-fold higher than normal activity of c-yes was observed in 3 out of 5 colon carcinoma cell lines. A 5-fold higher than normal activity was found in 10 out of 21 primary colon cancers, compared to normal colonic cells.

Selection of Cognate Transgene for Preparation of Cellular Immunogen

According to the present invention, a transgene construct is engineered comprising a transgene which is cognate to the target proto-oncogene (hereinafter "cognate transgene" or "CTG"). The transgene is selected such that it encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene. The transgene should be expressed to very high levels in the transfectants. Thus, the construct should contain a strong promoter.

The product encoded by the cognate gene must have a high degree of sequence homology with the product of the target proto-oncogene, but also must display some amino acid differences with the target proto-oncogene product. Thus, there must be a subset of one or more amino acid differences between the target proto-oncogene and its cognate in order to provide immunogenic stimulus.

Two classes of genes that satisfy these criteria are retroviral oncogenes and xenogenic proto-oncogenes. The word "xenogenic" is intended to have its normal biological meaning, that is, a property or characteristic referring or relating to a different species. Thus, a xenogenic proto-oncogene is meant to include the a homologous proto-oncogene of a species other than the host organism species. It may be appreciated that in the case of a target proto-oncogene, e.g. MDM2, for which no retroviral homolog is yet known, a xenogenic homologue is advantageously utilized as the source of the DNA for the cognate transgene.

In principle, a more effective immunogenic stimulus would depend on the particular sequence, and not on the distinction between a retroviral oncogene and a xenogenic proto-oncogene in terms of their relative transforming capacity. Thus, in certain cases, a retroviral oncogene may be better at providing a tolerance-breaking immunogenic stimulus, and in other cases, a xenogenic proto-oncogene may be more effective.

The retroviral oncogene or xenogenic proto-oncogene DNA forming the CTG may comprise the wild type oncogene or proto-oncogene DNA. More preferably, a mutant DNA is utilized, which is engineered so as to be non-transforming in the host. The DNA is mutated to include one or more nucleotide insertions, deletions or substitutions which will encode an oncogene product which is nontransforming in the host, but retains the requisite degree of sequence homology with respect to the target proto-oncogene. A cognate transgene deletion mutant (hereinafter "dCTG") is preferred.

A protein sequence is generally considered "cognate" with respect to the target proto-oncogene-encoded protein if it is evolutionarily and functionally related between species. A more precise view of cognation is based upon the following sequence comparison carried out utilizing the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference. Cognation is attained upon satisfying two criteria imposed by FASTA: (i) alignment of segments corresponding to at least 75% of the target proto-oncogene's encoded amino acid sequence; (ii) at least 80% amino acid identity within the aligned sequences. The segments of the target proto-oncogene protein sequence and protein test sequence satisfying the two criteria are referred to as "homology regions". Accordingly, at least 75% of the target proto-oncogene protein sequence is alignable with the test sequence. The alignable segments or homology regions may, however, represent less than 75% of the total test polypeptide chain for the case of test sequences that may significantly exceed the target proto-oncogene protein in length.

One skilled in the art, armed with the FASTA program, may survey existing sequence data bases (either protein sequences or DNA sequences, insofar as the amino acid sequence is determined by FASTA for all reading frames) for test sequences which are cognate with respect to the target proto-oncogene. At the same time, one can isolate and then sequence what are very likely to be cognate test sequences (*e.g.* feline MDM-2, as likely to be cognate to human MDM-2) and use FASTA to verify the presumed cognation, according to the criteria set forth above. One may obtain the sequences of presumptive cognate proto-oncogenes from a large number of mammalian sequences and screen these sequences with FASTA according to the aforesaid formulation of cognation.

Because the product encoded by a CTG differs at a small number of amino acid positions from the product encoded by the target proto-oncogene, an immunogenic stimulus is provided that (i) is directed against the foreign protein and (ii) with a lower probability, induce an anti-self response. The CTG is selected such that the gene product will yield the greatest immunogenic stimulus to induce anti-self reactivity. Provided that overall sequence homology (preferably greater than about 75%) is maintained, the presence of scattered amino acid differences is desired, since any one residue would likely have a relatively low probability of inducing self-reactivity. Moreover, the greatest number of residue differences would be advantageous, consistent with maintaining the requisite degree of general sequence homology.

The selection of amino acid modifications for the CTG may be facilitated by resort to available computer-based models used to identify immunogenic peptide fragments of polypeptides. These models could be employed to select CTGs which would possess the maximum number of immunogenic peptides for a given HLA haplotype.

Screening Procedure for CTG Selection

Notwithstanding the availability of computer-based algorithms which have some predictive value, it is desirable to design CTGs with resort to a screening procedure based on an actual experimental assay that can be HLA-haplotype specific. Accordingly, cells are biopsied from a normal volunteer of particular haplotype. The cells are transfected with a CTG construct, preferably a dCTG construct, satisfying the criteria set for cognition. More preferably, the cells are transfected with multiple dCTGs, preferably at least five dCTGs, satisfying the criteria for cognition. The at least five dCTGs are selected to display amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The transfected cells are then used to immunize the volunteer in accordance with the immunization method of the present invention. After immunization, the human subject is tested in a standard delayed hypersensitivity (DH) reaction with 10^4 - 10^6 irradiated, autologous fibroblasts, as transfected with the same dCTG (or series of dCTGs) as used for the immunizing preparation. A positive DH reaction (induration) would verify the induction of reactivity. The induction of reactivity in this assay is readily demonstrable because of the priming to the non-self determinants on the dCTG-encoded protein and the readout in the DH reaction of the same nonself determinants. Once DH reactivity is demonstrated in a DH reaction that directly tests the antigenicity of the non-self determinants encoded by the dCTG (*i.e.*, priming with a non-self construct, DH testing with the same non-self construct), the subject can be then tested in a DH reaction based on testing with the autologous cells transfected with a dCTG derived from the human proto-oncogene itself (*i.e.*, priming with a non-self construct, testing with the human self construct). Testing of a battery of human volunteers will lead to a catalogue of HLA-matched dCTGs, such that, for individuals of the same HLA haplotype, the use of the particular dCTG would be inductive of reactivity to proto-oncogene-encoded self. Different CTGs may thus be tested so as to correlate maximal secondary stimulation with a particular HLA haplotype.

At the same time, this procedure may be used with patients undergoing tumor resection (if post-operative immuno-suppressive protocols are not mandatory), such that prior to resection, a course of immunization would have been initiated, the endpoint of which would represent the development of a DH reaction.

Any given amino acid difference between the CTG-encoded product and the proto-oncogene-encoded product has a low probability of being a "tolerance-breaker". Thus, it is preferable to transfect the host cells with a mixture of multiple different CTGs, preferably dCTGs. The number of different dCTGs is preferably five or more. Moreover, it is preferred that, among themselves, the multiple dCTGs show amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The dCTGs would be selected to maximize amino acid differences and, at the same time, make sure that differences are found all along the polypeptide chain. It would thus not be preferable to select a battery of deletions all from within the same domain of the polypeptide chain.

According to a protocol which utilizes 10^7 irradiated cells for immunization containing five separate dCTGs, five groups of 2×10^6 cells are included in one inoculate, each group of 2×10^6 having been transfected with a separate dCTG from the total set of five CTGs that are cognate to a particular proto-oncogene.

Selection of Non-Transforming Cognate Transgenes

Non-transforming cognate transgene variants are most advantageously derived via deletion of a sequence essential for transformation. Unlike point mutations which are potentially reversible due to back mutations, deletion mutations are irreversible. Furthermore, deletion mutations do not possess the inherent disadvantage attaching to point mutations, namely, even though the requirement for generation of an acceptable cognate transgene is for a qualitative difference with the wild type, i.e., non-transforming versus

transforming, any given point mutation may be neutral or else quantitative in its effect, that is, the mutation may reduce but not totally eliminate transformability. Thus, according to a preferred embodiment of the invention, a deletion is created in a region of the cognate transgene which encodes an amino acid sequence required for transformation. Consonant with non-transformability, the smallest deletion possible so as to leave intact the bulk of the antigenicity of the transgene product is selected.

The engineering of a cognate transgene deletion mutant that satisfies these criteria is facilitated by reports of structure-function relationship in oncogene-encoded proteins. Such reports serve to identify regions of oncoproteins that are essential for transformation, as opposed to regions which are either neutral or serve merely to modulate transformability. Although such reports are usually based on *in vitro* transformation assays, and are therefore independent of immune effects, these studies can be exploited to aid in the construction of non-transforming dCTGs for use in the practice of the present invention.

The deletion mutant is engineered to include at least a part of the region identified as critical for transformation. In those cases where essential amino acids have been identified, the deletion will span these residues. The engineering of any desired deletion can be readily accomplished by polymerase chain reaction (PCR) according to conventional PCR techniques, based upon the known nucleotide sequence of the unmutated cognate transgene.

The following describes a representative protocol for deriving a non-transforming dCTG of the smallest possible deletion, for use in the practice of the present invention. A test dCTG, engineered on the basis of known or ascertained transformation-specific domains, and driven by the strongest possible promoter, is used to transfect murine 3T3 cells. A sister culture of 3T3 cells is also transfected, with non-deleted CTG. Each CTG or dCTG cell culture is inoculated into nude mice, in the absence of any treatment to render the cells non-dividing. Those dCTGs which do not yield tumors in the mice even after

prolonged observation are then utilized as transgenes for the biopsied human cells which, upon transfection with the transgene, will serve as a cellular vaccine according to the practice of the present invention. The dCTGs are selected with the smallest deletion mutant consonant with non-transformability.

- 5 Some CTGs representing xenogenic proto-oncogenes may not be tumorigenic in the 3T3/nude mouse assay. For any such non-transforming CTG, it is not essential to generate a dCTG. However, even given non-tumorigenicity in nude mice, it may be desirable to opt for generation of a deletion mutant when the transgene is based upon a xenogenic proto-oncogene. In such cases, the
- 10 deletion would be engineered so as to remove the homologous region to that deleted in the particular dCTG that corresponds to the deletion in the corresponding retroviral oncogene dCTG.

- Even though the transgene construct may comprise mutant oncogene or proto-oncogene DNA which is nontransforming, it is nevertheless
- 15 preferable, as a safety measure, to treat the transfected donor cells to render them non-dividing before inoculation into the host. The cells are irradiated with a radiation dosage sufficient to render them non-dividing.

Oncogenicity Assay of Cognate Transgenes

- As a further safety measure, the oncogenicity of a given dCTG is
- 20 preferably thoroughly tested prior to infection of the donor cells which are used as cellular immunogens according to the practice of the present invention. For example, an oncogenicity testing regimen may take the form of three separate assays: (i) dCTG transfection of NIH 3T3 cells, followed by inoculation into nude mice; (ii) dCTG transfection of human fibroblasts, followed by inoculation
- 25 into nude mice; and (iii) dCTG transfection of human fibroblasts, followed by an *in vitro* test of anchorage-dependent growth. In principle, all three should be negative to validate the use of any given dCTG in the vaccination method of the present invention.

According to the oncogenicity assay (i), after stable transfection of NIH 3T3 cells with the test dCTG, the transfectants are inoculated into nude mice. Tumorigenicity of the transfectants in the mice is then evaluated according to standard protocols.

5 According to oncogenicity assay (ii), human fibroblasts are transfected with the test dCTG as proposed in the above human immunization protocol. After stable dCTG transfection of human fibroblasts, however, rather than carrying out X-irradiation of the transfectants to render them non-dividing, followed by inoculation of the irradiated transfectants into the human host, the
10 transfectants are directly inoculated into nude mice as a direct test of tumorigenicity. Given the greater susceptibility of murine 3T3 cells to oncogenic transformation, *vis a vis* primary human or murine transfectants fibroblasts, assay (ii) is probably much less sensitive than assay (i), but does have the advantage of offering a direct test of dCTG oncogenicity in human cells.

15 According to oncogenicity assay (iii), non-irradiated dCTG-transfected human fibroblasts are assayed for anchorage-dependent growth, *i.e.* colony formation in soft agar, as a test of dCTG transforming potential in human cells. Anchorage independence, as defined by the ability of cells to grow when suspended in semisolid medium, is a common phenotype acquired by human
20 tumor cells, particularly those tumor cells of mesenchymal origin, such as fibrosarcomas. While assay (iii) has no *in vivo* readout, it offers an independent test of the critical issue of dCTG oncogenicity in human cells.

The oncogenicity assays are performed according to published protocols. Assay (i), comprising dCTG transfection of NIH 3T3 cells followed
25 by inoculation into nude mice, may be performed according to the protocol of Stevens *et al.*, *Proc. Natl. Acad. Sci. USA* (1988), 85:3875-3879, including DNA transfection by the calcium phosphate coprecipitation method of Manohaven *et al.*, *Carcinogenesis* (1985), 6:1295-1301. Accordingly, NIH 3T3 cells (7.5×10^5 cells per 100-mm dish) are exposed to a calcium phosphate-DNA
30 coprecipitate (40 μ g of genomic DNA plus 3 μ g of pSV2neo per dish) for 4

hours. Two days later, each dish is trypsinized and reseeded into a 175-cm² flask. For the next 10 days, cultures are selected in G418 (400 µg/ml), and the flasks are then trypsinized and cells are replated in the same flask to disperse the G418-resistant colonies into a diffuse lawn of cells. Two days later, the cells are harvested and washed with serum-free medium prior to injection. One injection of 5 X 10⁶ cells into the right flank and one injection of 1 X 10⁷ cells into the left flank, each in a volume of 200 µl, are done on each nude mouse. Injection sites are monitored at 3- or 4-day intervals for 100 days. The sites are scored for the number of tumors induced per injection site.

Oncogenicity assay (ii), whereby dCTG transfection of human fibroblasts followed by inoculation into nude mice, is carried out in the same manner as assay (i) except that for assay (ii) the human fibroblast transfectants are substituted for the murine 3T3 transfectants.

Assay (iii), involves a test of the *in vitro* anchorage-dependent growth of dCTG-transfected human fibroblasts. The assay is carried out as described in Stevens *et al.*, *J. Cancer Res. and Clin. Oncol.* 1989, 115:118-128. 1 x 10⁵ cells are seeded per 60-mm dish into 0.33% Noble agar over a 6-ml 0.5% agar base layer in Hams F10 supplemented with 6% fetal bovine serum. A portion of the agar suspension is diluted with Hams F10 plus 6% fetal calf serum to 200 cells/5 ml to determine the cloning efficiency of these cells when seeded into plastic 60-mm dishes. Agar dishes are fed with 1 ml Hams F10 supplemented with 6% fetal bovine serum on the 1st and 15th day after seeding. Four weeks after seeding, all agar colonies > 75 µm in diameter are counted and the colony counts are normalized to the plating efficiencies which aliquots of the initially seeded cells showed on plastic. This comparison, or normalization, of the agar colony counts to the plastic dish colony counts is useful in identifying and correcting for any mechanical artifacts which might result from the seeding into agar of dead cells that had persisted from the initial transfection treatment or from heat-induced cell death, which might have occurred while suspending cells in molten agar during the process of seeding the agar dishes.

The following is a partial list of various deletions which, based upon published accounts of experiments with human or animal cells, are believed to render the identified CTG non-tumorigenic.

Table 2
Deletion Mutations Rendering Indicated Gene Non-Transforming

| CTG | Genbank accession number for sequence | Number of amino acids in gene | Amino acids deleted, rendering CTG non-transforming | References |
|-------------------------------|---|-------------------------------|---|--|
| <i>Akt-2</i> (c-akt) (mouse) | M95936; SEQ ID NO:3 (<i>Mus musculus</i> serine/threonine kinase) | 480 | 148-234 | Bellacosa <i>et al.</i> , <i>Science</i> (1991), 254:274-278; Bellacosa <i>et al.</i> , <i>Oncogene</i> (1993), 8(3):745-54. |
| <i>c-neu</i> (c-erbB-2) (rat) | M11730; SEQ ID NO:4 (human tyrosine kinase-type receptor (HER2) gene) | 1255 | 1-731 | Bargmann <i>et al.</i> , <i>EMBO</i> (1988), 7(7):2043-52; Bernards <i>et al.</i> , <i>Proc. Natl. Acad. Sci. USA</i> (1987), 84(19):6854-8. |

09744406.012201

| CTG | Genbank accession number for sequence | Number of amino acids in gene | Amino acids deleted, rendering CTG non-transforming | References |
|---------------|--|-------------------------------|---|---|
| mdm-2 (human) | U33199: SEQ ID NO:5 (human mdm2-A mRNA); U33200: SEQ ID NO:6 (human mdm2-B mRNA); U33201: SEQ ID NO:7 (human mdm2-C mRNA); U33202: SEQ ID NO:8 (human mdm2-D mRNA); U33203: SEQ ID NO:9 (human mdm2-E mRNA) | 489 | 9-155 | Dubs- Poterszman, <i>Oncogene</i> (1995), 11(11):244 5-50. |

09744400.012001

| CTG | Genbank accession number for sequence | Number of amino acids in gene | Amino acids deleted, rendering CTG non-transforming | References |
|---|--|-------------------------------|---|--|
| <i>c-myb</i> (human) | J02012; SEQ ID NO:10 (proviral oncogene <i>v-myb</i>) | 640 | 275-327 | Kalkbrenner <i>et al.</i> , <i>Oncogene</i> (1990), 5(5):657-61. |
| <i>c-myc</i> (human) | X00364; SEQ ID NO:11 (human <i>c-myc</i> oncogene) | 439 | 129-144 | Sarid <i>et al.</i> , <i>Proc. Natl. Acad. Sci. USA</i> (1987), 84(1):170-3. |
| <i>v-ras</i> (Harvey Murine Sarcoma Virus) | M77193; SEQ ID NO:12 (Rat sarcoma virus <i>v-ras</i> oncogene) | 189 | 32-44 | Zhang <i>et al.</i> , <i>Science</i> (1990), 249:162-5 (1990) |
| <i>v-src</i> (Rous Sarcoma Virus) | U41728; SEQ ID NO:13 (RSV Schmidt-Ruppin A clone SRA-V; <i>v-src</i> gene) | 526 | 430-433 | Bryant <i>et al.</i> , <i>Mol. Cell. Bio.</i> (1984), 4(5):862-6. |

09744406.012201

| CTG | Genbank accession number for sequence | Number of amino acids in gene | Amino acids deleted, rendering CTG non-transforming | References |
|----------------------------|---|-------------------------------|---|---|
| c- <i>yes</i> (chicken) | D00333. SEQ ID NO:14 (human c- <i>yes</i> -2 gene) | 541 | 438-441 | Zheng <i>et al.</i> ; <i>Oncogene</i> (1989), 4(1):99-104. |

Engineering of Vectors for Donor Cell Transfection

The engineering of vectors for expression of a particular CTG, preferably a dCTG, is based on standard methods of recombinant DNA technology, *i.e.* insertion of the dCTG via the polylinker of standard or commercially available expression vectors. The dCTG is operably linked to a strong promoter. Generally speaking, a "strong" promoter is a promoter which achieves constitutively high expression of the dCTG in the transfected cells. Each promoter should include all of the signals necessary for initiating transcription of the relevant downstream sequence. These conditions are fulfilled, for example, by the pBK-CMV expression vector available from Stratagene Cloning Systems, La Jolla, CA (catalog no. 212209). The pBK-CMV vector contains the cytomegalovirus (CMV) immediate early promoter. dCTGs xenogenic with respect to a particular target proto-oncogene may be isolated by conventional nucleic acid probing techniques, given the availability of a highly homologous probe represented by the cognate retroviral oncogene and/or the human proto-oncogene itself.

Donor Cells for Transfection

The allogeneic donor cells which may be transfected to derive the cellular immunogens of the present invention must express class I MHC and be susceptible to isolation and culture. Fibroblasts express class I MHC and may

be cultured. Other preferred donor cells are bone marrow-derived antigen-presenting cells such as macrophages, follicular dendritic cells, and Langerhans cells, for example. The cells may comprise primary explants or established cell lines.

- 5 Primary skin fibroblasts may be obtained as follows. Punch biopsies can be performed by a competent physician as a standard clinical procedure. Each biopsy yields a starting population of $1-2 \times 10^7$ cells that would proliferate in culture. Methods for the preparation of tissue cultures of human fibroblasts are well developed and widely used. See, Cristofalo and Carpenter,
10 *J. Tissue Culture Methods* (1980), 6:117-121, the entire disclosure of which is incorporated herein by reference. Essentially, skin obtained by punch biopsy is washed using an appropriate wash medium, finely minced and cultured in a suitable culture medium, such as Dulbecco's Modified Eagle Medium (DMEM), under CO_2 at 37°C . The cells are trypsinized with a trypsin solution and
15 transferred to a larger vessel and incubated at 37°C in culture fluid.

Donor Cell Transfection

- The expression vector carrying the dCTG is used to transfect allogeneic donor cells according to conventional transfection methods. One method of transfection involves the addition of DEAE-dextran to increase the
20 uptake of the naked DNA molecules by a recipient cell. See McCutchin and Pagano, *J. Natl. Cancer Inst.* (1968) 41:351-7. Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of Ca^{++} to a phosphate-containing DNA solution. The resulting precipitate apparently includes DNA in association with calcium phosphate crystals. These
25 crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfectant, as well as in its clonal descendants. See Graham *et al.*, *Virology* (1973), 52:456-467 and *Virology* (1974), 54:536-539.

Preferably, transfection is carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) or related liposome-forming materials. See Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7417 (DNA-transfection); Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989), 86:6077-6081 (RNA-transfection). One preferred technique utilizes the LipofectAMINE™ Reagent (Cat. No. 18324-012, Life Technologies, Inc., Gaithersburg, MD) which is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Chemical Abstracts Registry name: N-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxypentyl)amino}ethyl)-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate), and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. Transfection utilizing the LipofectAMINE™ Reagent is carried out according to the manufacturer's published protocol. The protocol (for Cat. No. 18324-012) provides for either transient or stable transfection, as desired.

The advantage of transient expression is its rapidity, *i.e.* there is no requirement for cellular proliferation to select for stable integration events. This rapidity could conceivably be of major clinical importance, in cases of an already metastatic tumor burden, wherein the weeks required for selection of stable transfectants may simply not be available to the clinician.

There are, nonetheless, two general disadvantages to the use of transient transfection. The first is that expression usually peters out after a few days, in contrast to the continual expression in the case of stable transfection. This is not particularly crippling in terms of our immunization protocol. The inoculated, irradiated cells used for immunization would likely not survive *in vivo* for more than 4 or 5 days, in any case. (For the same reason, immunological rejection of the allogeneic donor cells by the host is not a concern). Thus the nominal advantage accruing to stable transfection, that of a long-duration expression by the progeny of the parental inoculated cell, is not of particular

relevance in the case of the immunizing regime described herein, which is based on the use of non-dividing, probably short-lived cells.

3 A second disadvantage of transient transfection resides in the fact
4 that it yields a cell population, only a subset of which has actually been
5 transfected and thus expresses the protein encoded by the transgene. This
6 problem is obviated in the case of stable transfection, wherein over time one can
7 develop a pure population of transfectants via selection for a resistance marker,
8 such as *neo*, under conditions of clonal proliferation of the initial stable
9 transfectants. *i.e.* daughter cells of transiently transfected cells lack the transgene.
10 in contrast to the case with stable transfectants. In the situation where there is
11 sufficient time to effect immunization based on stably transfected cells, the
12 progeny of all transfected clones would be utilized, not just the progeny of a
13 single clone, as is sometimes done for detailed biochemical and molecular
14 analyses of gene expression. Clearly the more clones utilized, the more quickly
15 one can arrive at the requisite number of cells to be used for immunization.

Percentage of Cells Exhibiting dCTG Expression

1 The percentage of cells exhibiting dCTG expression may be
2 determined by an immunohistology assay. In this procedure, a small number of
3 cells (~500) from the harvested pellet following centrifugation of transfected cells
4 are deposited on a cover slip and fixed with cold acetone. At this point, a
5 standard immunohistological assay is carried out with the cells on the cover slip.
6 *i.e.* addition of a primary monoclonal antibody reactive to the dCTG-encoded
7 protein, followed by the addition of a developing antibody, *e.g.* a fluorescent
8 tagged antibody reactive to the primary monoclonal antibody. Measurement of
9 the percentage of cells scoring as dCTG-positive in the fluorescent assay allows
10 a determination of the number of positive transfectants in the starting culture, and
11 thus the number of total cells to be used for immunization to arrive at the desired
12 number of dCTG-positive cells to be inoculated in the patient.

- If, as would be almost certain, the percentage of cells scoring as dCTG-positive is less than one hundred percent, one can simply increase the number of cells to be used for immunization, so as to include the desired number of transfectants. The non-transfected cells in the immunizing population would
- 5 simply represent x-irradiated, autologous fibroblasts that would constitute no danger to the patient.

Transfectant Irradiation

- Prior to inoculation into the host, the transfected cells are preferably irradiated. The transfectants are irradiated with a radiation dose
- 10 sufficient to render them non-dividing, such as a dose of 25 Gy or 2500R. The cells are then counted by trypan blue exclusion, and about 2×10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

Vaccination Procedure

- 15 The transfected cells are inserted into the host to achieve vaccination.

- It is the object of the present invention to generate a systemic tumor immune response, so as to fight metastasis formation wherever any metastases are found. Intramuscular, intraperitoneal or subcutaneous inoculation
- 20 will suffice to yield a systemic response. Thus, patients are vaccinated with the transfected cells accordingly.

- For *s-crc* overexpression associated with colon carcinoma, partial venous inoculation is preferred, as the liver is a frequent site of metastases. For vaccinating against breast cancers and lymphomas, systemic immunization is
- 25 preferred.

As a general rule, it is desirable to generate the strongest immune response consistent with clinical monitoring of no adverse side effects, *i.e.* multiple rounds of inoculation with, for example 10^7 cells, at each round. The

- number of rounds of inoculation is selected accordingly. The efficacy of the inoculation schedule may be monitored by a delayed hypersensitivity reaction administered to the patient. A course of about up to 10 inoculations, at 2-3 week intervals, may be utilized. It may be appreciated that the inoculation schedule
- 5 may be modified in view of the immunologic response of the individual patient, as determined with resort to the delayed-type hypersensitivity (DTH) reaction.

Patient Response Monitoring by Delayed-type Hypersensitivity Reaction

- Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction. DTH has been used clinically
- 10 (Chang *et al.* (1993), *Cancer Research* 53:1043-1050). To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 cells in a volume of 0.1 ml Hanks buffered saline solution (HBSS) are inoculated intradermally into the host. Induration is measured 48 hours later, as an average of two perpendicular diameters (responses of greater than ≥ 2 mm is considered positive).

- 15 One advantage to the DTH assay is that it can independently assess the induction of T cell reactivity to (i) the transfectants used for immunization (*i.e.* the set of 5 or more dCTGs chosen for immunization purposes, each containing non-self determinants) and (ii) transfectants, as transfected with the human dCTG itself containing only self determinants. Thus, the induction of
- 20 reactivity to the transfectants used for immunization establishes that the immunizing transfectants are in fact immunogenic, that is, the patient has not exhibiting a much weakened capacity for immune response. If the patient is demonstrably capable of response to the immunizing transfectants, then skin testing with the dCTG (human) transfectants would establish whether or not
- 25 reactivity to the human proto-oncogene encoded product had been induced. According to the practice of the invention, inoculation of the immunizing transfectants would continue for at least as long as the induction of reactivity to the human proto-oncogene-encoded protein occurs.

The practice of the invention is illustrated by the following nonlimiting examples.

Example 1

Immunization of Chickens Against c-src(527)-Induced

5

Tumors By Vaccination with v-src DNA

A. Genes

The oncogene *c-src(527)* is an activated form of chicken *c-src*. Its protein product pp60^{c-src(527)} differs from the protein product of *c-src*, pp60^{c-src}, by only a single amino acid substitution, phenylalanine for tyrosine at residue 527 (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73). This substitution eliminates the negative regulatory influence exerted on pp60^{c-src} phosphokinase activity by the enzymatic phosphorylation of the position 527 tyrosine. The protein product of *v-src*, pp60^{v-src}, shows a number of sequence differences with pp60^{c-src} (Takeya and Hanafusa, (1983) *Cell* 32, 881-890), including scattered single amino acid substitutions within the first 514 residues and a novel C terminus of 12 amino acids (residues 515-526), in place of the nineteen C terminal amino acids of pp60^{c-src} (residues 515-533). Both the *v-src*-positive plasmid, pMvsrc, and the *c-src(527)*-positive plasmid, pcsrc527, were originally shown (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73) to transform murine NIH 3T3 cells in culture. However, the *v-src*-induced transformants exhibited a more rapid or more extensive colony growth in soft agarose than the *c-src(527)*-induced transformants, as well as a usually shorter latency of tumor formation in nude mice (*id.*).

B. Plasmids

1. pVSR-C1

The pVSR-C1 plasmid was prepared as described by Halpern *et al.*, (1991) *Virology* 180, 857-86. Essentially, the plasmid was derived from the pRL^v-src plasmid (Halpern *et al.*, (1990) *Virology* 175, 328-331) by subcloning the v-src(+) *Xho*I-*Eco*RI fragment of the latter into the multiple cloning sequence of pSP65 (Melton *et al.*, (1984) *Nucleic Acids Res.* 12, 7035-7056) which had been cleaved with *Sal*I and *Eco*RI; since ligation of the *Xho*I overhang at the *Sal*I site destroys both recognition sequences, subsequent removal of the v-src(+) insert from the vector was achieved by digestion with *Eco*RI and with *Hind*III, which cleaves at a position in the multiple cloning sequence adjacent to the *Sal*I site. The pVSR-C1 plasmid was restricted with *Eco*RI and *Hind*III, so as to liberate the tumorigenic insert. This insert included the v-src oncogene of the subgroup A strain of Prague RSV, as flanked downstream by a portion of the long terminal repeat (LTR) of RSV (from the 5' start of the LTR, to the single *Eco*RI site).

2. pMvsrc

The pMvsrc plasmid was generously provided by Dr. David Shalloway, Cornell University, Ithaca, NY. The plasmid is prepared according to Johnson *et al.*, (1985) *Mol. Cell. Biol.* 5, 1073-1083. Briefly, the 3.1-kb *Bam*HI-*Bg*/II Schmidt Ruppin A v-src fragment from plasmid pN4 (Iba *et al.*, (1984) *Proc. Nat. Acad. Sci. USA* 81, 4424-4428) is inserted into the pEVX plasmid (Kriegler *et al.*, (1984) *Cell* 38,483-491) at a *Bg*/II site lying between two Moloney murine leukemia virus (MoMLV) long terminal repeats (LTRs). This fragment contains 276 bp of pBR322 DNA from the pBR322 *Bam*HI to *Sal*I sites followed by 2.8 kb of Rous sarcoma virus (RSV) DNA from the *Sal*I site that is about 750 bp upstream of the *env* termination codon down to the *Nru*I site that is about 90 bp downstream of the v-src termination codon. (The *Nru*I site

is converted to a *Bgl*II site in the construction of pN4.) Ligation is performed by using a 10:1 insert-vector DNA fragment molar ratio.

- The pMvsrc plasmid was restricted with *Nhe*I, so as to liberate a tumorigenic fragment. The fragment included the *v-src* oncogene of the subgroup A strain of Schmidt-Ruppin RSV, as flanked upstream by most of the Moloney murine leukemia virus (MoMLV) LTR (from the *Nhe*I site near the 5' start of the LTR, to the 3' end of this LTR) and downstream by a small portion of the MoMLV LTR (from the 5' start to the *Nhe*I site).

3. pcsrc527

- The pcsrc527 plasmid is prepared according to Kmiecik and Shalloway, (1987) *Cell* 49, 65-73. Briefly, a plasmid is constructed by cleaving expression vector pEVX (Kriegler *et al.*, (1984) *Cell* 38,483-491 at its unique *Bgl*III site lying between two MoMLV LTRs and inserting the 3.2 kilobase (kb) pair *Bam*HI-*Bgl*III hybrid *src* fragment from plasmid pHB5 in the proper orientation. This fragment contains sequences from pBR322, the SRA *env* 3' region, SRA *v-src*, *src* from recovered ASV, and chicken *c-src*. The *Bgl*III site is generated by insertion of a linker at the *Sac*I site about 20 bp downstream from the *c-src* termination codon. The restriction map of pMHB5 contains the MoMLV splice donor about 60 bp downstream from the 3' end of the upstream LTR and the *v-src* splice acceptor about 75 bp upstream from the *src* ATG.

Plasmid pMHB5527 is constructed by inserting the synthetic double-stranded DNA oligomer

| | | |
|----|---|----|
| 5' | CCAGTTCCAGCCTGGAGAGAACCTATA (SEQ ID NO:1) | 3' |
| 3' | TCGGGGTCAAGGTCGGACCTCTCTTGGATATCTAG (SEQ ID NO:2) | 5 |

- into pMHB5 between the *Ban*II site at *c-src* codon 524 and the downstream unique *Bgl*III site. This alters the TAC Tyr 527 codon to a TTC Phe codon while preserving the remaining *c-src* coding region. Equimolar amounts of the double-stranded oligomer and three gel-purified tandem restriction fragments from

- 5 pMHB5 are ligated in one reaction, which contains the following: the oligomer with *Ban*II and *Bg*III complementary ends, the 3 kb *Bg*III-*Bg*II (*Bg*II in the pEVX ampicillin resistance gene) partial digest fragment, the adjacent 6.1 kb *Bg*II-*Bg*II (downstream *Bg*II in *c-src*) fragment, and the 0.38 kb *Bg*II-*Ban*II (*Ban*II at *c-src* codon 524) fragment.

- 10 Plasmid *pcsrc527* is constructed by replacing the 2 kb *Sal*I (in *env*)-*Mlu*I (in *c-src*) fragment in plasmid pMHB5527, with the homologous fragment from plasmid p5H. This fragment contains the coding sequence for the *c-src* amino region (codons 1 to 257) that have been isolated by molecular cloning of a *c-src* provirus and previously shown by sequencing to contain authentic *c-src* sequence without the mutation at codon 63 (Levy *et al.*, (1986) *Proc. Natl. Acad. Sci. USA* 83, 4228-4232). Equimolar amounts of complementary gel-purified *Sal*I-*Mlu*I fragments from p5H and the other plasmids are ligated.

- 15 The *pcsrc527* plasmid was restricted with *Nhe*I, so as to liberate a tumorigenic fragment. The tumorigenic fragment included the *c-src*(527) oncogene, as flanked by the same LTR complement as in pMvsrc.

C. Animals

- 20 Chickens of two closed lines. SC and TK, were utilized. These lines differ at the major histocompatibility (*B*) complex (B^2/B^2 for the SC line, B^{15}/B^{21} for the TK line). Embryonated eggs were obtained from Hyline International (Dallas Center, IA). All chickens were hatched at the University of New Hampshire Poultry Research Farm and housed in isolation.

D. Tumor Induction by Plasmid DNA

- 25 Tumors were induced by subcutaneous inoculation in the wing web of a *src*-positive plasmid according to the technique described by Fung *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80, 353-357 and Halpern *et al.*, (1990) *Virology* 175, 328-331. Of the three tumorigenic plasmids utilized here, all were

adjusted, prior to inoculation, to a concentration of 100 μ g of enzyme-restricted DNA per 100 μ l of phosphate-buffered saline. The conditions of inoculation used for particular experiments (age of chicken at time of inoculation, amount of plasmid, etc.) are indicated below.

5 E. Growth of Primary (wing web) Tumors in TK or SC Chickens Inoculated with pVSRC-C1, pMVsrc or pcsrc527

Individual 1-day-old chickens of line TK or of line SC were inoculated with 100 μ g of either pVSRC-C1, pMVsrc or pcsrc527. The mean tumor diameter (mm) at a particular time point and for any one group of TK or
10 SC line chickens inoculated with an individual src-positive construct was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The results are shown in Fig. 1A (line TK) and Fig. 1B (line SC). The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total
15 number of survivors to that point (standard typeface for pcsrc527, italics for pVSRC-C1, bold typeface for pMVsrc). Error bars (unless obscured by the symbol) indicate standard error.

F. Growth of Challenge (wing web) Tumors in Test and Control Line TK Chickens Under Conditions of Priming and Homologous Challenge with pcsrc527, or Priming and Homologous Challenge with pVSRC-C1

20

Growth of challenge (wing web) tumors in test and control line TK chickens was determined under conditions of (i) priming and homologous challenge with pcsrc527, or (ii) priming and homologous challenge with pVSRC-C1. Test chickens were primed at 1 day posthatch with 100 μ g of construct; test
25 and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge tumor diameter was computed as described in the preceding section. At each time point the ratio of chickens bearing palpable

challenge tumors to total number of survivors to that point is indicated for priming and homologous challenge with *pcsrc527* (Fig. 2A) and priming and homologous challenge with *pVSRC-C1* (Fig. 2B) (standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). The statistical comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test: the paired ratios are underlined for only those time points where $p < 0.05$. Error bars indicate standard error.

G. Growth of Challenge (wing web) Tumors in Test and Control line TK chickens under Conditions of Priming with *pVSRC-C1* and Heterologous Challenge with *pcsrc527*, or Priming with *pcsrc527* and Heterologous Challenge with *pVSRC-C1*

Growth of challenge (wing web) tumors in test and control line TK chickens, was determined under conditions of (i) priming with *pVSRC-C1* and heterologous challenge with *pcsrc527*, or (ii) priming with *pcsrc527* and heterologous challenge with *pVSRC-C1*. Test chickens were primed at 1 day posthatch with 100 μ g of construct; test and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge tumor diameter was computed as described in Section E. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming with *pVSRC-C1* and heterologous challenge with *pcsrc527* (Fig. 3A) and priming with *pcsrc527* and heterologous challenge with *pVSRC-C1* (Fig. 3B) (standard typeface for control group, bold typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described in the preceding section [*($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), for the student's t test], and the paired ratios are

underlined for only those time points where, in the chi-squared test, $p < 0.05$. Error bars indicate standard error.

H. Discussion

In a direct comparison of the growth of tumors induced in line TK by either pMvsrc or pVSR-C1, a similar pattern of relatively rapid regression was observed. This result established that the difference in LTR complement between these two v-src positive constructs did not exert a major influence on the tumor growth pattern in the TK line (Fig. 1A). By contrast, much more extensive and persistent tumor growth resulted from inoculation of TK chickens with the pcsrc527 construct (Fig. 1A). The relatively greater growth capacity of tumors induced by this construct indicated that in the TK line, the c-src(527) oncogene is much more highly tumorigenic than the v-src oncogene. This difference did not, however, generalize to the SC line (Fig. 1B). The SC line was chosen for comparison with the TK line on the basis of earlier observations (Halpern *et al.*, (1993) *Virology* 197, 480-484) that v-src DNA-induced tumors engender a much weaker tumor immune response in line SC than in line TK. Whereas the growth of pcsrc527-induced primary tumors was virtually indistinguishable in the two lines, the growth of the v-src-induced tumors was considerably greater in the SC than in the TK line (Figs. 1A and 1B). Thus v-src, but not c-src(527), gives rise to primary tumors whose growth patterns differ in the two lines analyzed here.

Only minimal protection against homologous challenge was observed under conditions of priming to c-src(527) DNA, indicative of the induction of a relatively weak tumor immune response (Fig. 2A; a statistically significant lowering of challenge tumor growth in the test versus the control chickens was observed at only one time point). By contrast, the v-src DNA-primed chickens showed excellent protection against the homologous tumor challenge (Fig. 2B).

- Priming with v-src DNA engenders a relatively greater degree of protection against challenge with c-src(527) DNA, than that afforded by priming with c-src(527) DNA itself (Fig. 3A). The degree of protection was weaker than that determined (Fig. 2B) for the case of priming and homologous challenge with v-src DNA. Only marginal protection was observed, however, when the heterologous challenge protocol was carried out in the reverse order (Fig. 3B). These results demonstrate that induction of reactivity to an antigenicity specified in tumor cells by an overexpressed proto-oncogene can confer tumor immunity.

Example 2

Vaccination Protocol

- The following is a representative vaccination protocol according to the present invention. While the protocol includes transfection of cells freshly harvested from appropriate donors, the donor cells could as easily be obtained from cultures housed in cell banks. In such a case, the protocol would commence directly with part C., Fibroblast Transfection.

A. Skin Punch Biopsy

A punch biopsy of skin is obtained from a donor (other than the patient-to-be-treated or identical twin thereof) by a trained physician following standard medical practice.

20 B. Preparation of Fibroblast Culture

- Under sterile conditions, the skin obtained by punch biopsy is put in a tube with 10 ml of the following wash medium: Dulbecco's Modified Eagle Medium (DMEM), containing sodium bicarbonate (30 ml/liter of a 5.6% solution) and penicillin/streptomycin (2 ml/liter of a pen-strep stock solution containing 5000 units penicillin and 5000 µg of streptomycin/ml, pH 7.2-7.4.). In a sterile hood, the skin biopsy is added to a Petri dish, and then transferred several times to new Petri dishes containing the same wash medium. The biopsy is then finely minced with two scalpels, and 2-4 pieces (<1 mm³) of the minced biopsied are placed in the middle part of one or more T25 flasks. The flask is

- placed in a tissue culture incubator at 37°C for one half hour with the cap firmly closed, then opened for 10 minutes. The following culture medium is prepared: DMEM containing sodium bicarbonate; antibiotics; and 10% fetal calf serum containing 2.5 µg/ml fungizone, 40 µg/ml gentamicin, and 1% glutamine (3% W/V). Two ml of the culture medium is then added to the flask, and the flask is incubated at 37°C (5% CO₂), with the cap lightly unscrewed. The flask is left for three days without moving so as to obtain adhesion of the separate pieces of skin to the plastic. Afterwards, the medium is changed two times per week over a 3-4 week period always adding 2-3 ml of medium. To trypsinize the skin cell culture, one needs zones of confluence. After aspirating the culture medium, 5 ml of the Puck's Saline A/EDTA solution (0.4 g EDTA to 1 liter of Puck's Solution A) is added and immediately aspirated. Then 1 ml of trypsin solution (0.05/0.02% trypsin in PBS, without Ca++ or Mg++) is added and incubated for 5 min at 37°C, at which time 2 ml of culture fluid is added to stop the action of the trypsin. The cells are then transferred to a larger flask (T75) and incubated at 37°C in 15 ml of culture fluid, which is changed every 2 days.

C. Fibroblast Transfection

- The fibroblasts (2 X 10⁵ cells) are washed twice in DMEM without serum or antibiotics. A LipofectAMINE™-DNA solution is prepared by mixing in tube #1 mix 400 µl DMEM and 10 µl of dCTG vector DNA (1 µg/ul). In tube #2, 400 µl DMEM and 25 µl of LipofectAMINE Reagent (Life Technologies, cat. no. 18324-012) are mixed. The contents of tube #1 and #2 are mixed together and are then left sitting at room temperature for 30 hours. Then, 3.2 ml of the LipofectAMINE™-DNA solution is added to the cells. The cells are incubated for six hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refed with growth medium and incubated for an additional 24 hours at 37°C.

D. Transfectant Irradiation

Transfectants are irradiated to a dose of 25 Gy or 2500R. the cells are then counted by trypan blue exclusion. 2×10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

5 E. Vaccination

Patients are vaccinated by subcutaneous inoculation of 2×10^7 irradiated cells at 2-3 week intervals. A shorter or longer regimen is used, depending upon the results of delayed type hypersensitivity (DTH) reaction monitoring (described below).

10 F. Patient Assessment by DTH Monitoring

Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction, as described by Chang *et al.* (1993). *Cancer Research* 53:1043-1050. To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 transfected irradiated cells in a volume of 0.1 ml HBSS are inoculated intradermally. Induration is measured 48 hours later, as an average of two perpendicular diameters. Responses of greater than 2 mm are considered positive.

Example 3v-myc Transfection of Murine Fibroblasts20 A. Vector Preparation

The v-myc retroviral oncogene of avian myelocytomatosis virus MC29 (Land *et al.* (1983). *Nature* 304:596-602) was obtained from the American Type Culture Collection, Rockville, MD. 20852, as the pSVv-myc vector (ATCC No. 45014). The v-myc-positive *EcoRI-KpnI* fragment of pSVv-myc was ligated into the polylinker sites of the pBK-CMV plasmid (Stratagene Cloning Systems, La Jolla, CA).

B. Cell Transfection

Stable transfection using the pBK-CMV-v-myc vector was carried out on a line of A31 fibroblasts (Balb/c origin), obtained from the ATCC. 2 X 10⁵ cells were seeded in a 100 mm/dish and allowed to grow for 18-20 h (RPMI 1640 medium and 10% fetal bovine serum), at which time the cells reached 50-70% confluence. The cells were then washed twice in Dulbecco's Modified Eagles Medium (without serum or antibiotics). A LipofectAMINE™-DNA solution was prepared according to Example 2.C., with the pBK-CMV-v-myc vector DNA, and 3.2 ml of the LipofectAMINE™-DNA solution added to the cells. The cells were then incubated for 6 hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refed with the growth medium and incubated for an additional 24 hour at 37°C. Thereafter, the cells were fed once every two days with growth medium containing 250 µg/ml geneticin (G418; Gibco BRL cat. no. 11811) as the selective marker. Within two weeks, colonies were picked and expanded into permanent cell lines. The cells were then washed and collected by centrifugation.

It should be noted that the procedure for transient transfection is the same, through the point of incubation with the Lipofectamine™-DNA solution. Thereafter, the cells are washed and incubated for 72 hours in growth medium.

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indication the scope of the invention.

Claims

1. A cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target
5 proto-oncogene is associated with a cancer, which cellular immunogen comprises allogeneic donor cells which have been transfected with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host
10 immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.
2. An immunogen according to claim 1 wherein the transgene comprises
wild-type or mutant retroviral oncogene DNA; or
15 wild-type or mutant proto-oncogene DNA of a species different from the host species.
3. An immunogen according to claim 2 wherein the transfected cells are non-dividing.
4. An immunogen according to claim 2 wherein the transgene
20 comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.
5. An immunogen according to claim 4 wherein the mutant DNA is nontransforming.

6. An immunogen according to claim 5 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

5 7. A cellular immunogen according to claim 6 wherein the donor cells have been transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

8. An immunogen according to claim 1 wherein the donor cells have been transfected with a transgene cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, *c-erbB-2*,
10 MDM-2, *c-myc*, *c-myb*, *c-ras*, *c-src* and *c-yes*.

9. An immunogen according to claim 1 wherein the donor cells comprise fibroblasts or bone marrow-derived antigen-presenting cells.

10. A method for preparing a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the
15 overexpression of which target proto-oncogene is associated with a cancer, the method comprising:

transfecting allogeneic donor cells with at
least one transgene construct comprising at least
one transgene cognate to the target proto-oncogene
and a strong promoter to drive the expression of
20 the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

- comprises
11. A method according to claim 11 wherein the transgene
- wild-type or mutant retroviral oncogene DNA; or
- wild-type or mutant proto-oncogene DNA of a species
- 5 different from the host species.
12. A method according to claim 11 wherein the transfected cells
- are non-dividing.
13. A method according to claim 11 wherein the transgene
- comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.
- 10 14. A method according to claim 13 wherein the mutant DNA is
- nontransforming.
15. A method according to claim 14 wherein the mutant DNA
- comprises a deletion mutation in a region of said DNA which is essential for
- transformation.
- 15 16. A method according to claim 15 wherein the donor cells are
- transfected with a plurality of transgene constructs, each construct encoding a
- different deletion mutation.
17. A method according to claim 11 wherein the transgene is
- cognate to a target proto-oncogene selected from the group of proto-oncogenes
- 20 consisting of AKT-2, *c-erbB-2*, MDM-2, *c-myc*, *c-myb*, *c-ras*, *c-src* and *c-yes*.
18. A method according to claim 1 wherein the donor cells
- comprise fibroblasts or bone marrow-derived antigen-presenting cells.

0944405 012201
103210 9044405

19. A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene comprising

(a) transfecting allogeneic donor cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene;

(b) inserting the cells transfected with the transgene construct into the body of the host to obtain expression of the transgene in the host.

20. A method according to claim 19 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or
wild-type or mutant proto-oncogene DNA of a species different from the host species.

21. A method according to claim 20 wherein the transfected cells are rendered non-dividing prior to insertion into the body of the host.

22. A method according to claim 20 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

23. A method according to claim 22 wherein the mutant DNA is nontransforming.

24. A method according to claim 23 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

25. A method according to claim 24 wherein the donor cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

26. A method according to claim 19 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, *c-erbB-2*, MDM-2, *c-myc*, *c-myb*, *c-ras*, *c-src* and *c-yes*.

27. A method according to claim 19 wherein the donor host cells comprise fibroblasts or bone marrow-derived antigen-presenting cells..

28. A method of vaccinating a host against disease associated with the overexpression of a targeted proto-oncogene comprising

(a) transfecting allogeneic donor cells with at least one transgene construct comprising at least one transgene and a strong promoter to drive the expression of the transgene in the transfected cells, wherein the transgene comprises

(1) wild-type or mutant cognate retroviral oncogene DNA; or

(2) wild-type or mutant cognate proto-oncogene DNA of a species different from the host species;

(b) inserting the cells transfected with the transgene construct into the body of the host to obtain expression of the transgene in the host.

09/744406

1/3

FIG. 1A

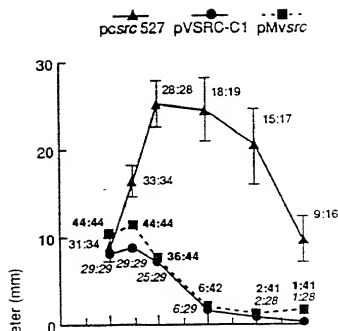


FIG. 1B

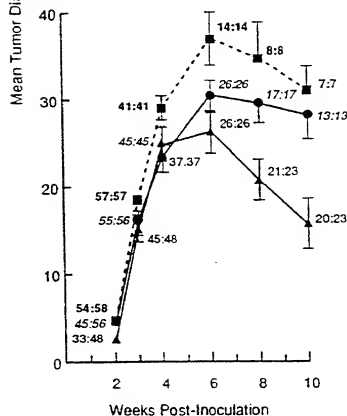


FIG. 2A

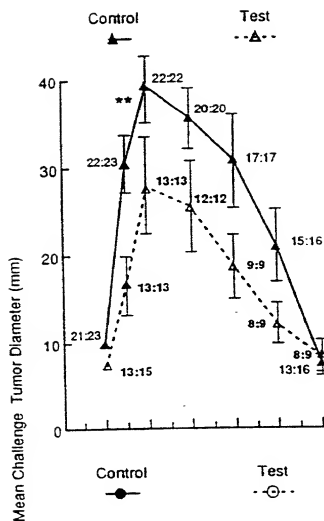
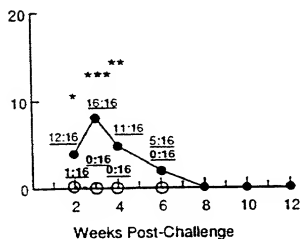


FIG. 2B



09/744406

3/3

FIG. 3A

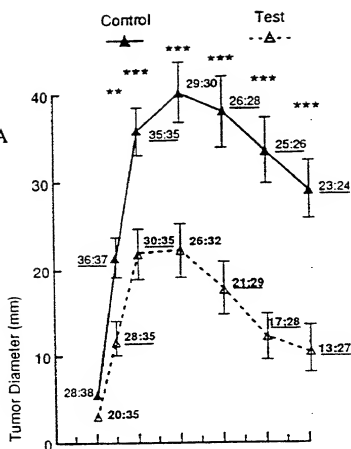
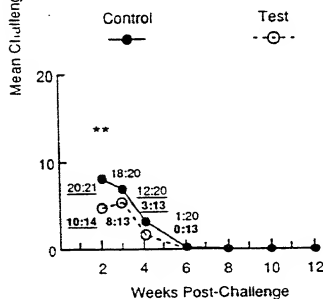


FIG. 3B



DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

ALLOGENEIC CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES

the specification of which is attached hereto unless the following box is checked

☒ was filed on _____ as Application Serial No. _____ or PCT Application No. PCT/US99/15594 and filed on July 8, 1999 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S)

| COUNTRY/OFFICE | APPLICATION NO. | DATE OF FILING | PRIORITY CLAIMED |
|----------------|-----------------|----------------|--|
| _____ | _____ | _____ | <input type="checkbox"/> YES <input type="checkbox"/> NO |
| _____ | _____ | _____ | <input type="checkbox"/> YES <input type="checkbox"/> NO |

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER**DATE OF FILING**

60/093,965

JULY 24, 1998

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS
DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120**

Status (check one)

| Application Serial No. | Date of Filing | Patented | Pending | Abandoned |
|------------------------|----------------|--------------------------|--------------------------|--------------------------|
| _____ | _____ | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| _____ | _____ | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

And I hereby appoint Arthur H. Seidel, Registration No. 15,979; Gregory J. Lavorgna, Registration No. 30,469; Daniel A. Monaco, Registration No. 30,480; Thomas J. Durling, Registration No. 31,349; and John J. Marshall, Registration No. 29,671, my attorneys or agents with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all correspondence to Seidel, Gonda, Lavorgna & Monaco, P.C., Suite 1800, Two Penn Center Plaza, Philadelphia, Pennsylvania 19102. Address all telephone calls to Daniel A. Monaco, (215)568-8383 (telefax: 215-568-5549).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF SOLE OR FIRST INVENTOR

MICHAEL

S.

HALPERN

(GIVEN NAME)

(MIDDLE INITIAL OR NAME)

(FAMILY OR LAST NAME)

Inventor's signature: Michael S. Halpern

Date: 1/17/2001

Country of Citizenship: United States

USX

Residence: WEST NEWTON, MA

(City)

(State or Foreign Country)

Post Office Address: 66 Taft Avenue

West Newton, Massachusetts 02465 USA

FULL NAME OF SECOND INVENTOR

JAMES

M.

ENGLEND

(GIVEN NAME)

(MIDDLE INITIAL OR NAME)

(FAMILY OR LAST NAME)

Inventor's signature: _____

Date: _____

Country of Citizenship: United States

Residence: _____

MEDIA

PENNSYLVANIA

(City)

(State or Foreign Country)

Post Office Address: 644 Rosetree Road

Media, Pennsylvania 19063

FULL NAME OF SOLE OR FIRST INVENTOR

MICHAEL

S.

HALPERN

(GIVEN NAME)

(MIDDLE INITIAL OR NAME)

(FAMILY OR LAST NAME)

Inventor's signature: _____

Date: _____

Country of Citizenship: United States

Residence: WEST NEWTON, MA

(City)

(State or Foreign Country)

Post Office Address: 66 Taft Avenue

West Newton, Massachusetts 02465 USA

FULL NAME OF SECOND INVENTOR

JAMES

M.

ENGLAND

(GIVEN NAME)

(MIDDLE INITIAL OR NAME)

(FAMILY OR LAST NAME)

Inventor's signature: _____

Date: 1-16-01

Country of Citizenship: United States

USX

Residence: _____

MEDIA

PENNSYLVANIA

(City)

(State or Foreign Country)

Post Office Address: 644 Rosetree Road

Media, Pennsylvania 19063

PCT

RAW SEQUENCE LISTING

PATENT APPLICATION: US/09/744,406

DATE: 04/25/2001

TIME: 07:32:27

Input Set : A:\Halpern.app

Output Set: N:\CRF3\04252001\I744406.raw

ENTERED

```

3 <110> APPLICANT: Halpern, Michael S
4      England, James M.
5      Allegheny University of the Health Sciences
7 <120> TITLE OF INVENTION: Allogeneic Cellular Immunogens Useful As Cancer
8      Vaccines
10 <130> FILE REFERENCE: 7933-38 PC
C--> 12 <140> CURRENT APPLICATION NUMBER: US/09/744,406
C--> 13 <141> CURRENT FILING DATE: 2001-01-22
15 <150> PRIOR APPLICATION NUMBER: 60/093,965
16 <151> PRIOR FILING DATE: 1998-07-24
18 <160> NUMBER OF SEQ ID NOS: 14
20 <170> SOFTWARE: PatentIn Ver. 2.0
22 <210> SEQ ID NO: 1
23 <211> LENGTH: 27
24 <212> TYPE: DNA
25 <213> ORGANISM: Artificial Sequence
27 <220> FEATURE:
28 <223> OTHER INFORMATION: Description of Artificial Sequence: One strand of
29      db1-stranded oligomer for altering c-src codon 527
31 <400> SEQUENCE: 1
32      ccagttccag cctggagaga acctata                               27
34 <210> SEQ ID NO: 2
35 <211> LENGTH: 35
36 <212> TYPE: DNA
37 <213> ORGANISM: Artificial Sequence
39 <220> FEATURE:
40 <223> OTHER INFORMATION: Description of Artificial Sequence: One strand of
41      db1-stranded oligomer for altering c-src coden 527
43 <400> SEQUENCE: 2
44      gatctatagg ttctctccag gctggaactg gggct                               35
46 <210> SEQ ID NO: 3
47 <211> LENGTH: 1599
48 <212> TYPE: DNA
49 <213> ORGANISM: Mus musculus
51 <400> SEQUENCE: 3
52      gagactgtgc cctgtccaag gtgcctcctg catgtcctgc tgccctgagc tgtcccgagc 60
53      taggtgacag cgtaccacgc tgccaccatg aatgaggtgt ctgtcatcaa agaaggctgg 120
54      ctccacaagc gtggtgaata catcaagacc tggaggccac ggtacttctt gctgaagagc 180
55      gacggtctct tcattgggta caaggagagg ccgagggccc ctgatcacag tctacccccc 240
56      ttaaacaact ttctcgtagc agaatgccag ctgatgaaga ccgagaggcc ggcacccaac 300
57      acctttgtca tacgctgcct gcagtggacc acagtcatcg agaggacott ccacgtggat 360
58      tctccagacg agagggagga gtggatgcgg gccatccaga tggtcgccaa cagcctcaag 420
59      cagcgggccc caggcgagga ccccattggc tacaagtgtg gctccccccg tgactctccc 480
60      acgactgagc agatggaagt ggcggtcagc aaggcacggg ctaaaagtgc catgaatgac 540
61      ttccagatgc taaaactcct tggcaaggga acotttggca aagtcattct ggtcggggag 600
62      aaggccaact gccgctacta gcgcatgaag atcctgcgaa aggaagtcat cattgccaa 660
63      gatgaagtc ctcacacagt caccgagagc cgggtctctc agaacaccag gcacccgttc 720

```

RAW SEQUENCE LISTING
PATENT APPLICATION: US/09/744,406

DATE: 04/25/2001
TIME: 07:32:27

Input Set : A:\Halpern.app

Output Set: N:\CRF3\04252001\I744406.raw

```

64 ctaactgcgc tgaagtatgc cttccagacc caccgaccgc tgtgctttgt gatggagtat 780
65 gccaacgggg gtgagctgtt cttccacctg tccggggagc gtgtcttcac agaggagcgg 840
66 gcccggtttt atggtgcaga gattgtctcg gctcttgagt acctgcactc gccgggacgtg 900
67 gtataccgcy gaatacaagc ggaaaacctc atgtcgaca aagatggcca catcaagatc 960
68 actgactttg gctctgtcaa agagggcato agtgacgggg ccaccatgaa aacctctctg 1020
69 ggggaccccg agtacctggc gctgagggtg ctggaggaca atgactatgg gccggccgtg 1080
70 gactgggtgg ggtgggtgtt ggtcatgtac gagatgatgt gcggccgcct gccctctcac 1140
71 aaccaggacc acgagcgctt cttcgagctc atcctcatgg aagagatccg cttcccgctg 1200
72 acgtccagcc ccgagggcaa gtccctgctt gctgggctgc ttaagaagga ccccaagcac 1260
73 aggettgtgt gggggcccaag cgaatgccaag gaggatcatg agcaccaggtt ctctctcagc 1320
74 atcaactggc aggcacttgg ccaagaagaag ctctgccacc ctttcaaacg tcaggtcacg 1380
75 tccgagggtg acacaaggta cttogatgat gaatttaacc ccagttccat cacaatacca 1440
76 cccctgacc gctatgacag cctgggtcta ctggagctgg accagcggaag ccaettcccc 1500
77 cagttctctc actcgccgag catccgcgag tgagcagttt gccccgcgag aggcgcgacg 1560
78 ctgcgtgcca tcaccgctgg gtggtttttt acccctgcc 1599

80 <210> SEQ ID NO: 4
81 <211> LENGTH: 4530
82 <212> TYPE: DNA
83 <213> ORGANISM: Human
84 <400> SEQUENCE: 4

36 aattctcgag ctgctgacc ggtcgacgag ctcgagggtc gacgagctcg agggcgcgcg 60
37 cccggccccc accctctgca gcaaccgcgg ccccgccccc tcccagccgg gtccaagccg 120
38 agccatgggg ccggagccgc agtgagcacc atggagctgg cggcctgttg ccgctggggg 180
39 ctccctctcg cctcttggc ccccgagacc gggagcacc aagtgtgcac cggcaccagc 240
40 atgaagctgc ggtcctctgc cagtcccgag accacactgg acatgctcgc ccacctctac 300
41 cagggtcgc aggttggtgca gggaaacctg gaactcaact aactgcccac caatgcagc 360
42 ctgtctcttc tgacagatat ccaggagggtg cagggtcagc tgctcatgca tcaacaacca 420
43 gtgaggcgag tccactgca gaggctgcgg attgtgcgag gcaaccagct cttttaggac 480
44 aactatgccc tggcctgtgt agacaatgga gaaccgctga acaataccac cctctgtaac 540
45 ggggcctccc caggagcctt gcgggagctg cagcttgtaa gctcaacaga gatcttgaaa 600
46 ggaggggtct tgatccagct gaacccccag ctctgctacc aggacacgat tttgtgaa 660
47 gacatctctc acaagaacaa ccagctggct ctcaactgta tagacaccaa ccgctctcgg 720
48 gctctgcaac cctgttctcc gatgtgtaag ggctccgctg gctggggaga gatttctgag 780
49 gattgtcaga gcttgacggc cactgtctgt gccggttgct gtgcccgctg caagggggcca 840
100 ctgcccactg actgtgcca tgagcagttg gctgccggtt gcaaggggcc caagcactct 900
101 gctgctctgg cctgtctcca cttcaaccac agtggcatct gtgagctgca gtccccagcc 960
102 ctggtgacct acaacacaga cagcttttag tccatgccca atcccagggg ccggtataca 1020
103 ttcggtcagc cgtgtgtgac tgctgtcccc tacaactacc tttctacgga cttgggatac 1080
104 tgcacctcgt tctgccccct gcacaaccaa gaggtgacag cagaggatgg aaacaagcgg 1140
105 tgtgagaagt gcagcaagcc ctgtgcccga gtgtgctatg gtcgggctatg gtaggcactt 1200
106 cgagagggtg gggcagttac cagtgccaat atccaggagt ttgtctggctg caagaagatc 1260
107 tttgggagcg tggcattttt gccggagagc tttgatgggg acccagcctc caaacctgoc 1320
108 cgcgtccagc cagagcagct ccaagtgttt gagactctgg aagagatcac aggttaacta 1380
109 tacatctcag catggccgga cagcctgcct gacctcagcg tcttcacaga cctgcaagta 1440
110 atccggggac gaattctgca caatggcgcc tactcgctga ccttgcgaag cctgggcatc 1500
111 agctggctgg ggtgcgtctc actgagggaa ctggggcagtg gaactggcct catcoacat 1560
112 aacacccacc tctgctctgt gcacaagggt cctggggacc agctcttttg gaaccgcgac 1620
113 caagctctgc tccacactgc caaccggcca gaggacaggt gtgtggcgga ggccctggcc 1680
114 tgcaccagcg tgtgcccggc agggcaactgc tgggttccag ggcccaccca gtgtgtcaac 1740

```

RAW SEQUENCE LISTING

DATE: 04/25/2001

PATENT APPLICATION: US/09/744,406

TIME: 07:32:27

Input Set : A:\Halpern.app

Output Set: N:\CRF3\04252001\I744406.raw

```

115 tgcagccagt tcccttgggg ccaggagtg cgtggaggaa gccgagta cgcaggggctc 1800
116 cccaggagat atgtgaatgc caggcaactgt ttgccgtgcc accctgagtg tcagccccaag 1860
117 aatgggtcag tgacctgttt tggaccggag gctgaccagt gtgtggcgctg tgcocactat 1920
118 aaggaccctc cctctctcgt gccccgctgc cccagcggtg tgaaacctga cctctcctac 1980
119 atgcocatct ggaagtttcc agatgaggag ggcgcattgc agccttgccc catcaactgc 2040
120 accocatctt gtgtggacct ggatgacaa ggtctccccc ccgagcagag agccagccct 2100
121 ctgacgtcca tegtctctgc ggtggttgcc attctgctgg tcgtggtctt gggggtggtc 2160
122 tttgggatcc tcatacagcg accgcagcag aagatccgga agtacaagat gcggagactg 2220
123 ctgcaggaaa cggagctggt ggagcgcgtg acacctagcg gagcgatgcc caaccaggcg 2280
124 cagatgcgga tcttgaaga gacggagctg aggaaggtga aggtgctgtg atctggcgct 2340
125 tttggcaacg tctacaaggg catctggatc cctgatgggg agaattgtga aattccagtg 2400
126 gccatcaaag tgttgaggga aaacacatcc cccaagcca acaagaaat cttagacgaa 2460
127 gcatacgtga tggctggtgt gggctcccca tatgtctccc gccttcttgg catctgcctg 2520
128 acatccacgg tgcagctggt gacacagctt atgcccattg agacctgttc 2580
129 cgggaaaaac gcggcagcct gggctccagg gacctgtgta actgtgtgat cgagattgcc 2640
130 aaggggatga gtaacctgga ggaatgtggg ctogtacaca gggacttgaa attccagtg 2700
131 gtgctgtgta agagtcccaa ccatgtcaaa attacagact tcgggctggc tcggctgctg 2760
132 gacattgacg agacagagta ccatgcagat gggggcaagg tgccccaa gttggtggcg 2820
133 ctgagtgata ttctcgcgc gcggttcacc caccagagt atgtgtggag ttatgtgtg 2880
134 actgtgtggg agctgtgac ttttggggcc aaacctta cgtggatccc agcccgagg 2940
135 atccctgacc tgcgtgaaaa gggggagcgc ctgccccagc ccccactgtg caccattgat 3000
136 gtctacatga tcatggtcaa atgttggatg attgactctg aatgtcggcc aagattccgg 3060
137 gagttggtgt ctgaattctc ccgcattggc agggaccccc agcctcttgg gtcactccag 3120
138 aatgaggact tgggcccagc cagtcccttg gacagacct tctaccgctc actgctggag 3180
139 gacgatgaca tgggggacct ggttgatgct gaggagtatc tggtaaccca gcagggtctc 3240
140 ttctgtccag accctgcccc gggcgctggg ggcattgctc accacaggca ccgacgtcca 3300
141 tctaccagga gtggcggttg gaaactgaca ctagggtctg agccctctga agaggaggcc 3360
142 cccagctctc cactggcacc ctccgaagg gctggctccc atgtatttga ttgtgacctg 3420
143 ggaatggggg cagccaagg gctgcaaa cccccacac atgacccag cctctacag 3480
144 cggtagcagt aggaacccac agtacccttg cctctgaga ctgtaggcta cgttgcccc 3540
145 ctgacctgca gccccaagcc tgaatatgtg aaccagccag atgttcggcc ccagccccct 3600
146 tcgccccgag agggccctct gctgtctgcc cgacctgctg gtgccaactt ggaaggggcc 3660
147 aagactctct cctcggaggaa gaatggggtc gtcaaaagac tttttccct tgggggtgcc 3720
148 gtggagaacc ccgagtactt gacacccagc ggaggagctg cccctcagcc ccacccctct 3780
149 cctcgctcca gccacacct cgaacacctc tattactggg accagagccc accagagcgg 3840
150 ggggctccac ccagcacctt caaagggaca cctacggcag agaaccaga gtacctgggt 3900
151 ctgagcgtgc cagtgtgaac cagaaggcca agtcgcaga agcctctgat tgttcctcag 3960
152 gacgaggaaa ggcctgactt ctgctggcat caagagtggt gaggggcctc cgaccacttc 4020
153 caggggaacc tgcacgtcca ggaacctgtc ctaaggaaac ttcctctcct ctgagtcttc 4080
154 ctgagtgctg gaagggttcg agcctcgttg gaagagaaac agcactgggg agtctttgtg 4140
155 gattctgagg cctgcccaa tgagactcta gggctccagt gatgccacg ccagcttgg 4200
156 ccccttccct ccagatctgt ggtactgaaa gccttaggga agctggcctg agaggggaag 4260
157 cgcocctaca ggaagtgtcta agaaacaaa gcacccattc agagactgtc cctgaaacct 4320
158 agtactgccc ccatgagga aggaacagca atggtgtcag tatccaggct ttgtacagag 4380
159 tttctttctg tttagttttt actttttttg ttttgtttt ttaaagacga aataaagacc 4440
160 caggggagaa tggggtgtgt atggggaggc aagtgtgggg ggtccttctc cacaccact 4500
161 ttgtccattt gcaaatatat ttggaaaac 4530
162 <210> SEQ ID NO: 5
163 <211> LENGTH: 891

```

RAW SEQUENCE LISTING
PATENT APPLICATION: US/09/744,406

DATE: 04/25/2001
TIME: 07:32:27

Input Set : A:\Halpern.app

Output Set: N:\CRF3\04252001\I744406.raw

```

165 <212> TYPE: DNA
166 <213> ORGANISM: Human
168 <400> SEQUENCE: 5
169 atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
170 gcttcggaac aagagaccct ggactttgat gctggtgtaa gtgaacatct aggtgattgg 120
171 ttgtgacagg attcagtttc agatcagttt agtgtagaat ttgaagttga atctctcgac 180
172 tcagaagatt atagccttag tgaagaagga caagaacctc cagatgaaga tgatgaggtg 240
173 tatcaagtta ctgtgtatca gccagggggg agtgatacag attcatttga agaagatcct 300
174 gaaatttcct tagctgacta ttggaaatgc acctcatgca atgaaatgaa tccccccctt 360
175 ccatcacatt gcaacagatg ttggcccctt cgtgagaatt ggcttctctg agataaaggg 420
176 aaagataaag gggaaatctc tgagaaagcc aaactggaaa actcaacaca agctgaagag 480
177 ggctttgatg ttctctgatt taaaaaaact atagtgaatg attccagaga gtcattgtgt 540
178 gaggaaaaat atgataaaat tacacaagct tcacaatcac aagaagtga agactattct 600
179 cagccatcaa ctctctagat cattatttat agcagccaa gaaagtgaag agagtttgaa 660
180 aggggaagaa cccaagacaa agaagagagt gtggaatcta gtttgccctt taatgccatt 720
181 gacgttgctg tgatttgtca aggtcgacct aaaaatggtt gcatttgtcca tggcaaaaca 780
182 ggacatctta tggcctgctt tacatgtgca aagaagctaa agaaaaggaa taagccctgc 840
183 ccagtatgta gacaaaccaat tcaaatgatt gtgctaaact atttccccta g 891
185 <210> SEQ ID NO: 6
186 <211> LENGTH: 657
187 <212> TYPE: DNA
188 <213> ORGANISM: Human
190 <400> SEQUENCE: 6
191 atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
192 gcttcggaac aagagaccct ggactatttg aaatgcaact catgcaatga aatgaatccc 120
193 ccccttccat cacattgcaa cagatgttgg gcccttcgtg agaattggct tcttgaagat 180
194 aaaggggaaa ataaagggga aatctctgag aaagccaaac tggaaaactc aacacaagct 240
195 gaagagggct ttgatgttcc tgattgtaaa aaaactatag tgaatgattc cagagagtca 300
196 tgtgttgagg aaaaatgatg taaaattaca caagcttcac aatcaacaag aagtgaagac 360
197 tattctcagc catcaacttc tagtagcatt atttatagca gccaaagaag tgtgaaaagag 420
198 ttgaaagggg aagaaaaccaca agacaaagaa gagagtgtgg aatctagtgt gccctttaat 480
199 gccattgaac ctctgttgat ttgtcaaggt cgacctaaaa atggttgcat tgtccatggc 540
200 aaacacaggac atcttatggc ctgctttata tgtgcaagaa agctaaagaa atggaataag 600
201 ccttgcccag tatgtagaca accaattcaa atgattgtgc taacttattt cccctag 657
203 <210> SEQ ID NO: 7
204 <211> LENGTH: 966
205 <212> TYPE: DNA
206 <213> ORGANISM: Human
208 <400> SEQUENCE: 7
209 atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
210 gcttcggaac aagagaccct ggttagacca aagccattgc ttttgaagtt attaaagtct 120
211 gttgggtgac aaaaagcacac ttatactagt aaagaggatc ttgatgctgg tgaagtgaag 180
212 attcagggtg attggtttga tcaggattca gtttcagatc agtttagatg agaatgtgaa 240
213 gttgaatctc tcgactcaga agattatagc cttagtgaag aaggacaaga actctcagat 300
214 gaagatgatg aggtatatca agttactgtg tatcaggoag gggagagtga tacagattca 360
215 ttgaaagaag atcctgaaat ttctttagct gactattgga aatgcaactc atgcaatgaa 420
216 ataatcccc cccttccatc acattgcaac agatgttggg ccccttcgtg gaattggctt 480
217 cctgaagata aagggaaaga taaaggggaa atctctgaga aagccaaact gaaaaacta 540
218 acacaagctg aagagggctt tgatgttctt gattgtaaaa aaactatagt gaatgattcc 600

```


RAW SEQUENCE LISTING
PATENT APPLICATION: US/09/744,406

DATE: 04/25/2001
TIME: 07:32:27

Input Set : A:\Halpern.app

Output Set: N:\CRF3\04252001\I744406.raw

```

219 agagagtgcat gtgttgagga aaatgatgat aaaattacac aagcttcaca atcacagaag 660
220 agtgaagact attctcagcc atcaactctt agtagcatta tttatagcag ccaagaagat 720
221 gtgaagaagt ttgaagaagg aaaaacccaa gacaaagaag agagtgtgga atctagtgtg 780
222 ccccttaatg ccattgaacc ttgtgtgatt tgtcaaggtc gacctaaaaa tggttgcatt 840
223 gtccatggca aaacaggaca tcttatggcc tgcctttacat gtgcaagaag gctaaagaaa 900
224 aggaataaag cctgcccagt atgtagacaa ccaattcaaa tgattgtgct aactatttcc 960
225 ccctag
226
227 <210> SEQ ID NO: 8
228 <211> LENGTH: 399
229 <212> TYPE: DNA
230 <213> ORGANISM: Human
231
232 <400> SEQUENCE: 8
233 atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
234 gcttcggaac aagagaccct ggttagacca gaaagtgaag actattctca gccatcaact 120
235 tctatgata ttatttatag cagccaagaa gatgtgaag agtttgaaag ggaagaacc 180
236 caagacaaag aagagagtgt ggaatctagt ttgccctta atgccattga acctgtgtg 240
237 attgtcaag gtgcacctaa aaatggttgc attgtccatg gcaaaacagg acatcttatg 300
238 gctctgctta catgtgcaaa gaagctaaag aaaaggaata agccctgccc agtatgtaga 360
239 caaccaattc aaatgattgt gctaacttat tcccctag
240
241 <210> SEQ ID NO: 9
242 <211> LENGTH: 309
243 <212> TYPE: DNA
244 <213> ORGANISM: Human
245
246 <400> SEQUENCE: 9
247 atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
248 gcttcggaac aagagaccct ggttagacca aagccattgc ttttgaagt attaaagtct 120
249 gttgtgtgac aaaaagacac ttatactatg aaagaggttc tttttatct ttgccagtat 180
250 attatgacta aacgattata tgatgagaag caacaacata ttgtaaatg ttgtgctaac 240
251 ttatttcccc tagttgacct gtctataaga gaattatata tttctaacta tataacccta 300
252 ggaatttag
253
254 <210> SEQ ID NO: 10
255 <211> LENGTH: 1897
256 <212> TYPE: DNA
257 <213> ORGANISM: Avian myeloblastosis virus
258
259 <400> SEQUENCE: 10
260 cacagataag gttattttgg taccctctcg aaaagttaaa ccggacatcg cccaaaagga 60
261 tgaggtgact aagaagaagt aggcgagccc tctttttgca ggctggaggc acatagataa 120
262 gagaattatc actctacatt catctttctc aaagattaat ctactgtgtg gttttatat 180
263 tcattagaat cggacagatg ttcagtgcca gcaccggtgg cagaaagtat taaccagca 240
264 acttaacaaa gttccatgga ctaaaagaga ggtacaaaag gtaatagaac acgtgcagaa 300
265 atacggtcca aagcgctggt cggacattgc taagcatttg aagggaagga ttggaaaaca 360
266 gtgcagggag aggtggcaca accatctgaa tccagaagtg aagaaaaact ccggtgacaga 420
267 agagaaagat agaatatttt accaggcaca caagagactg ggaacacagat gggcagaaat 480
268 tgcaaaagtg ctgcctgagc ggaactgataa cgctgtcaag aaccaactgga attccacat 540
269 gcgcgcggag gtgcagcagg aggggttacc gcaggagtcc tccaaagccc gccgcgcctc 600
270 ggcaaacccc ggcttcacga agagcagcca tctgatggcc ttgcccaca accacactgc 660
271 aggcccgctc gccgggggccc gccaggcccc tctgggcagt gactaccctc attaccacat 720
272 gtctgagcca caaaatttcc ctggtcagat cccatatcca gtgacactgc atataaat 780
273 tatcaatggt cctcagccag ctgtgtcagc tattcagaga cactatactg atgaagacc 840

```

VERIFICATION SUMMARY

PATENT APPLICATION: US/09/744,406

DATE: 04/25/2001

TIME: 07:32:28

Input Set : A:\Halpern.app

Output Set: N:\CRF3\04252001\I744406.raw

L:12 M:270 C: Current Application Number differs, Replaced Application Number

L:13 M:271 C: Current Filing Date differs, Replaced Current Filing Date

04/25/01 07:32:28

SEQUENCE LISTING

<110> Halpern, Michael S
England, James M.
Allegheny University of the Health Sciences

<120> Allogeneic Cellular Immunogens Useful As Cancer
Vaccines

<130> 7933-38 PC

<140>
<141>

<150> 60/093,965
<151> 1998-07-24

<160> 14

<170> PatentIn Ver. 2.0

<210> 1
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: One strand of
dbl-stranded oligomer for altering c-src codon 527

<400> 1
ccagttccag cctggagaga acctata 27

<210> 2
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: One strand of
dbl-stranded oligomer for altering c-src codon 527

<400> 2
gatctatagg ttctctccag gctggaactg gggct 35

<210> 3
<211> 1599
<212> DNA

<213> Mus musculus

<400> 3

gagactgtgc cctgtccacg gtgectcctg catgtcctgc tgccctgagc tgtecccgagc 60
 taggtgacag cgtaccacgc tgccaccatg aatgaggtgt ctgtcatcaa agaaggctgg 120
 ctccacaagc gtggtgaata catcaagacc tggaggccac ggtacttctt gctgaagagc 180
 gacggctcct tcattgggta caaggagagg cccgaggccc ctgatccagc tctaccccc 240
 ttaaacact tcctcgtagc agaatgccag ctgatgaaga ccgagaggcc gcgacccaac 300
 acctttgtca taacctgcct gcagtggacc acagtcctgc agaggacctt ccacgtggat 360
 tctccagacg agaggggagg gtgatgcgg gccatccaga tggctgcgaa cagcctcaag 420
 cagcggggcc caggcgaggga ccccatggac tacaagtgtg gctccccagc tgaactcctc 480
 acgactgagg agatggaagt ggcgggtcagc aaggcacggg cttaaagtgc catgaatgac 540
 ttogactatc tcaaaactcct tggcaaggga acctttggca aagtcatcct ggtgcgggag 600
 aaggccactg gccctacta cgccatgaag atcctgcgaa aggaagtcac cattgccaa 660
 gatgaagtgc ctacacagc caccgagagc cgggtcctcc agaaccaccg gcacccgttc 720
 ctcaactgcg tgaagtatgc ctccagacc cagaccgcc tgtgctttgt gatggagtat 780
 gccaacgggg gtgagctgtt ctccacctg tcccgggagc gtgtcttcac agaggagcgg 840
 gcccggtttt attgtgcaga gattgtctgc gctcttgagt acttgcactc gcgggacgtg 900
 gtataccgag acatcaagct ggaaaacctc atgctggaca aagatggcca catcaagatc 960
 actgactttg gccctcgcga agagggcac agtgacgggg ccaccatgaa aacctctgt 1020
 gggaccccg agtacctggc gcctgaggtg ctggaggaca atgactatgg ccggggccgtg 1080
 gactgggtgg ggtctgggtg ggtcatgtac gagatgatg gcggcccgct gccctctac 1140
 aaccagagcc accgagccct ctccagctc atcctcatgg aagagatccg ctcccgccg 1200
 acgctcagcc ccgaggccaa gtccctgctt gctgggctgc ttaagaagga ccccaagcag 1260
 aggtctggtg gggggcccag cgatgccaa gaggctcatg agcacaggtt ctctctcagc 1320
 atcaactggc aggacgtggt ccagaagaag ctctgcac ccctcaaac cttaggtcac 1380
 tccgaggtgc acacaaggtt ctccgatgat gaatttacc cccagtcac cacaatcaca 1440
 cccccgacc gctatgacag cctgggctta ctggagctg accagcggac ccacttcccc 1500
 cagttctctc actcggccag catccgcgag tgagcagttc gccccacgag aggacgcagc 1560
 ctgcgtgcca tcaccgctg gtgtgttttt accctcgc 1599

<210> 4

<211> 4530

<212> DNA

<213> Human

<400> 4

aattctcgag ctctcgacc ggtcgacgag ctcgagggtc gacgagctcg agggcgccg 60
 ccggccccc accctcgca gcaccccgcc ccccgccgc tccagccgg gtccagccgg 120
 agccatgggg ccggagccgc agtgagcacc atggagctgg cggccttgtg ccgctggggg 180
 ctctctctcg cctcttgcc ccccgagcc gcgagcacc aagtgtgcac cggcacagac 240
 atgaagctgc ggctcctgc cagtcccgag acccaactgg acatgctccg ccacctctac 300
 cagggtctgc aggtgtgtga gggaaacctg gaactcacct acctgcccac caatgccagc 360
 ctgtccttc tgacgatat ccaggaggtg cagggtctac gtctcatcgc tcacaccaa 420
 gtgaggcagg tccactgca gaggctgcgg attgtgcgag gcacccagct ctttgaggac 480
 aactatgcc tggccgtgct agacaatgga gaacctgtga acaataccac cctgttcaca 540
 ggggctctcc caggagcct gcgggagctg cagcttcgaa gcttcacaga gctcttgaaa 600
 ggaggggtct tgatccagc gaacccccag ctctgtctacc aggcacagat tttgtggaag 660

gacatcttcc acaagaacaa ccagctggct ctcacactga tagacaccaa ccgctctcgg 720
 gcctgccacc cctgttctcc gatgtgtaag ggctcccgct gctggggaga gagttctgag 780
 gattgtcaga gctgacagcg cactgtctgt gccggtggct gtgcccgtgt caaggggcca 840
 ctgcccaact actgctgcc ttagcagtggt gctgccggt gccggggccc caagcactct 900
 gactgectgg cctgcctcca cttaaccac agtggcatct gtgagctgca ctgcccagcc 960
 ctggtcacct acaacacaga cagctttgag tccatgccca atccccaggg ccggtatata 1020
 ttccggcgca gctgtgtgac tgcctgtccc tacaactacc ttctacgga cgtgggatcc 1080
 tgcacctcg tctgcccct gcacaaccaa gaggtgacag cagaggatgt aacacacggg 1140
 tgtgagaagt gcagacagcc ctgtgccoga gtgtgctatg gtctggcatg ggagcaettg 1200
 cgagaggtga gggcagttac cagtgccaat atccaggagt ttgctggctg caagaagatc 1260
 tttgggagcc tggcatttct gccggagagc tttgatggg acccagctc caacactgcc 1320
 ccgctccagc cagagcagct ccaagtgtt gagactctgg aagagatcac aggttacctta 1380
 tacatctcag catggccgga cagcctgcct gacctcagcg tcttccagaa cctgcgaagta 1440
 atccggggag gaattctgca caatggcgcc tactcgctga cctgcgaagg cctgggcatc 1500
 agctggctgg ggctggtctc actgagggaa ctgggagtg gactggccct catccaccat 1560
 aacacccacc tctgtctcgt gcacacgggt cctggggacc agctcttctg gaaccggcac 1620
 caagctctgc tcccaactgc caaccggcca gagcagcagt gtgtggcgga ggctctggcc 1680
 tgcaccagcg tgtgcccgc agggcaactgc tgggggtccag gggccaccca gtgtgtcaac 1740
 tgcagccagt tcttctgggg ccaggagtgc gtggaggaat gccagagtact gcaggggctc 1800
 cccagggagt atgtgaatgc caggcactgt ttgcctgccc accctgagtg tcagccccag 1860
 aatggctcag tgacctgttt tggaccggag gctgaccagt gtgtggcctg tgcacctat 1920
 aaggacctc ccttctcgtg ggcccgtgc cccagcgggt tgaacctga cctctccatc 1980
 atgccatct ggaatttccc agatgaggag ggcgcagtgc agcctggccc actcaactgc 2040
 acccaactct gtgtggacct ggatgacaag ggctgcccgc ccgagcagag agccagccct 2100
 ctgacgtcca tctgtctcgt ggtggttggc attctgctgg tctgtgtctt gggggtggtc 2160
 tttgggatcc tcatcaagcg accggcagcg aagatccgga agtaacagat gcggagactg 2220
 ctgcaggaaa cggagctggt gggccgctg acacctagcg gagcgatgcc caaccaggcg 2280
 cagatcgcca tctgaaga gaaggagctg aggaaggtga agtgtcttg atctggcgct 2340
 tttggcacag tctacaagg catctggatc cctgatgggg agaattgaa aattccagt 2400
 gccatcaaa gtgtgaggga aaacacatcc cccaaagcca acaagaanaa cttagacgaa 2460
 gcatacgtga tggctggtgt gggctcccca tatgtctccc gccttctggg catctgcctg 2520
 acatccacgg tgcagctggt gacacagctt atgccctatg gctgcctctt agaccatgc 2580
 cgggaaaaacc gcggacgct gggctcccag gacctgctga actggtgtat gcagattgcc 2640
 aaggggatga gctacctgga ggaatgtcgg ctctacaca gggacttggc cgtcgggaac 2700
 gtgctggcca agagtcccaa ccatgtcaaa attacagact tcgggctgctg tcggctgctg 2760
 gacattgacg agacagagta ccatgcagat gggggcaagg tgcccatcaa gtggatggcg 2820
 ctggagtcca ttctccgcg cgggttacc caccagagt atgtgtggag ttatggtgtg 2880
 actgtgtgga agctgatgac ttttggggcc aaaccttacg atgggatccc agccgggag 2940
 atccctgacc tgttgaaaaa gggggagcgg ctgcccagc ccccatctg caccattgat 3000
 gtctacatga tcatggtcaa atgttgatg attgactctg aatgtggcc aagattccgg 3060
 gagttgtgtg cgaattctc ccgcatggcc agggaccccc agcgtttgt ggtatccag 3120
 aatgaggact tgggcccagc cagtccctg gacagcaact tctaccgtc actgctggag 3180
 gacgatgaca tgggggacct ggtggtatgt gaggagatc tggtaaccca gcagggett 3240
 tctgttccag acctgcctcc gggcgctggg ggcattgctc accacaggca ccgcagctca 3300
 tctaccagga gtggcggtgg ggacctgaca ctagggtcgg agccctctga agaggagcc 3360
 cccaggtctc cactggcacc ctccgaagg gctggctcgg atgtatttga tggtagacct 3420
 ggaatgggtc cagccaaagg gctgcaaaag ctcccacac atgacccac cctctacag 3480
 cgttacagt aggacccac agtaccctg cctctgaga ctgatggta cgttggcccc 3540

```

ctgacctgca gccccagac tgaatatgtg aaccagccag atgttcggcc ccagccccct 3600
tcgccccgag agggccctct gcttgctgcc cgacctgctg gtgccactct ggaaagggcc 3660
aagactctct ccccaggga gaaatggggc gtcaaagacg tttttgcctt tgggggtgcc 3720
gtggagaacc ccgagtactt gacacccag ggaggagctg cccctcagcc ccacccctct 3780
cctgccttca gccagccctt cgacaacctc tattactggg accaggaccc accagagcgg 3840
ggggctccac ccagacacctt caaagggaca cctacggcag agaaccaga gtacctgggt 3900
ctggacgtgc cagtgtgaac cagaaggcca agtcgcgaga agccctgatg tgcctcagg 3960
gagcaggaag ggcctgacct ctgctggcat caagaggtgg gagggccctc cgaccacttc 4020
caggggaacc tgccatgccca ggaacctgtc ctaaggaaac ttccttctct cttgagtacc 4080
cagatggctg gaaggggctc agcctcgttg gaagaggaaac agcactgggg agtctttgtg 4140
gattctggag cctgcctcaa tgagaactca ggttcagctg gatgccacag cccagcttgg 4200
ccctttctct ccagatccctg ggtactgaaa gccttaggga agctggcctg agagggggaag 4260
cgcccttaag gtagtgtcta agaaccaaa cgacccattc agagactgtc cctgaaacct 4320
agtactggcc cccatgagga aggaacagca atggtgtcag tatccaggct ttgtacagag 4380
tgcttttctg tttagttttt actttttttg tttgttttt ttaaagacga aataaagacc 4440
caggggagaa tgggtgttgt atggggaggc aagtgtgggg ggtccttctc cacaccact 4500
ttgtccattt gcaaatatat tttggaaac 453C

```

<210> 5

<211> 891

<212> DNA

<213> Human

<400> 5

```

atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
gcttcggaac aagagacctt ggaacttgat gctgggtgaa gtgaacattc aggtgattgg 120
ttggatcagg attcagtttc agatcagttt agtgtagaat ttgaagttga atctctcgac 180
tcagaagatt atagccttag tgaagaagga caagaacctc catagtaaga tgatgaggtg 240
tatcaagtta ctgtgtatca ggcaggggag agtgatacag attcatttga agaagatcct 300
gaaatttctt tagctgacta ttggaatgc acttcatgca atgaaatgaa tccccccctt 360
ccatcacatt gcaacagatg ttggggccctt cgtgagaatt ggcttctctg agataaaggg 420
aaagataaag gggaaatctc tgagaaaagcc aaactggaaa actcaacaca agctgaagag 480
ggctttgatg ttctcgtattg taaaaaaact atagtgaatg attccagaga gtcattgttt 540
gaggaataat atgataaaat tacacaagct tcacaatcac aagaaagtga agactattct 600
cagccatcaa cttctagtag cattatttat agcagccaag aagatgtgaa agagtttgaa 660
agggaaagaa cccaagacaa agaagagagt gtggaactca gtttgccctt taatgccatt 720
gaaccttgtg tgatttgtca aggtcgacct aaaaatggtt gcattgtcca tggcacaaca 780
ggacatctta tggcctgctt tacatgtgca aagaagctaa agaaaaggaa taagccctgc 840
ccagtatgta gacaaccaat tcaaatgatt gtgtactact atttccctca g 891

```

<210> 6

<211> 657

<212> DNA

<213> Human

<400> 6

```

atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
gcttcggaac aagagacctt ggactattgg aaatgcacct catgcaatga aatgaatccc 120

```

```

ccccctccat caccattgcaa cagatgttgg gcccttcgtg agaattggct tccatgaagt 180
aaaggggaaa ataaagggga aatctctgag aaagccaaac tggaaaaact aacacaagct 240
gaagaggggt ttgatgttcc tgattgtaaa aaaactatag tgaatgattc cagagagtca 300
tgtgtgtgag aaaaatgatga taaaattaca caagcttcac aatcacaaaga aagtgaagac 360
tattctcagc catcaacttc tagtagcatt atttatagca gccaaagaaga tgtgaaagag 420
tttgaaaagg aagaaaccca agacaaagaa gagagtgtgg aatctagttt gccctttaat 480
gccattgaac cttgtgtgat ttgtcaaggc cgacctaaaa atggtttgat tgtccatggc 540
aaaacaggac atcttatggc ctgctttaca tgtgcaaaga agctaaagaa aaggaaataag 600
ccctgccacg tatgtagaca accaattcaa atgattgtgc taacttattt cccctag 657

```

<210> 7

<211> 966

<212> DNA

<213> Human

<400> 7

```

atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
gcttcggaac aagagacctt ggttagacca aagccattgc ttttgaagtt attaaagtct 120
gttgggtcac aaaaagacac ttatactatg aaagaggatc ttgatgtctg tgtaagttaa 180
cattcagggtc attggttggg tcaggattca gtttcagatc agtttagtgt agaatttgaa 240
gttgaattct cgactccaga agattatagc cttagtgaag aaggacaaga actctcagat 300
gaagatgatg aggtatatca agttactgtg tatcaggcag gggagagtga tacagattca 360
tttgaagaag atcctgaaat ttcccttagct gactattgga aatgcacttc atgcaatgaa 420
atgaatcccc cccctccatc acattgcaac agatgttggg ccccttcgtg gaattggctt 480
cctgaagata aagggaagaa taaaggggaa atctctgaga aagccaaact ggaataactca 540
acacaagctg aagagggtct tgatgttccg gattgtaaaa aaactatagt gaatgattcc 600
agagagtcac gtgttgaggg aaatgatgat aaaattacac aagcttcaca atcacaaaga 660
agtgaagact attctcagcc atcaactctt agtagcatta tttatagcag ccaagaagat 720
gtgaaagagt ttgaaaggga agaaacccaa gacaaagaag agagtgtgga atctagtttg 780
ccccctaatg ccatggaacc ttgtgtgatt tgtcaaggtc gacctaaaaa tggttgcatt 840
gtccatggca aaacaggaca tcttatggcc tgctttacat gtgcaaaaga gctaaagaaa 900
aggaataagc cctgccacgt atgtagacaa ccaattcaaa tgattgtgct aacttatttc 966
ccctag

```

<210> 8

<211> 399

<212> DNA

<213> Human

<400> 8

```

atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
gcttcggaac aagagacctt ggttagacaa gaaagtgaag actattctca gccatcaact 120
tctagtacga ttatttatag cagccaagaa gatgtgaaag agtttgaag ggaagaacc 180
caagacaaag aagagagttt ggaatctagt ttgcccttta atgccattga acctgtgttg 240
atttgtcaag gtgcacctaa aaatgggtgc atgttccatg gcaaaacagg acatcttatg 300
gctctgttta catgtgcaaa gaagctaaag aaaaggaata agccctgcc agtatgtaga 360
caaccaattc aaatgattgt gctaacttat ttcccctag 399

```

<210> 9
 <211> 309
 <212> DNA
 <213> Human

<400> 9
 atgtgcaata ccaacatgtc tgtacctact gatggtgctg taaccacctc acagattcca 60
 gcttcggaac aagagacctt ggttagacca aagccattgc ttttgaagtt attaaagtct 120
 gttggtgcac aaaaagacac ttatactatg aaagagggtc tttttatct tggccagtat 180
 attatgacta aacgattata tgatgagaag caacaacata ttgtaaatga ttgtgtcaac 240
 ttatttcccc tagttgacct gtctataaga gaattatata ttctaaacta tataacccta 300
 ggaatttag 309

<210> 10
 <211> 1897
 <212> DNA
 <213> Avian myeloblastosis virus

<400> 10
 cacagataag gttatttggg taccctctcg aaaagttaaa ccggacatcg cccaaaagga 60
 tgaggtgact aagaagaatg aggcgagccc tctttttgca ggctggaggc acatagataa 120
 gagaattatc actctacatt catctttctc aaagattaat ctacttgggt gttttatatt 180
 tcttagaat cg, 'cagatg ttcatgcca gcacgggtg cagaaaagtat taaccaccaga 240
 acttaacaaa ggtccatgga ctaaagagga ggatcaaagg gtaatagaac acgtgcagaa 300
 atacggtcca aagcgctggt cggacattgc taagcatttg aagggaaagga ttggaaaaca 360
 gtgcaggag aggtggcaca accatctgaa tccagaagtg aagaaaacct cctggacaga 420
 agagaaagat agaattattt accaggcaca caagagactg ggaacagat gggcagaaat 480
 tgcaaatgt ctgcctggac ggactgataa cgctgtcaag aaccactgga attccaccat 540
 gcgcgggaag gtcgcagcagg agggttaccc gcaggagctc tccaaagccg gccgcacctc 600
 ggcaaccacc ggcttccaga agagcagcca tctgatggcc ttgtcccaca acccactctg 660
 aggcgcctc ccgggggccc gccaggcccc tctgggcagt gactaccctc actaccacat 720
 tgcctagcca caaaatgtcc ctggtcagat cccatatcca gtagcactgc atataaatat 780
 tatcaatggt cctcagccag ctgctgcagc tattcagaga cactatactg atgaagacc 840
 tgagaaagaa aaacgaataa aggaattaga gttgctactt atgtgcagct agaatgaact 900
 gaagggcgag caggcatcac caacacagaa ccacacagca aactaccocg ctggcacag 960
 caccacgggt gctgacaata ccaggaccag tggtgacaat gcgcctgttt cctgtttggg 1020
 ggaacatcac cactgtactc catctccacc agtggatcat gggtgtctac ctgaggaaa 1080
 tgcgtcccc cccagggtgca tgattgttca ccagagcaac atcctggata atgttaagaa 1140
 tctcttagaa ttgcagaaa cactccagtt aatagactcc ttctaaaca catgtccaa 1200
 tcaagagaat ctgaacctgg acaacctgc actaacctcc acgccagtgt gtggccacaa 1260
 gatgtctgtt accaccccat tccacaagga ccagactttc actgaataca ggaagatgca 1320
 cggcggagca gtctagagct caattataat aatcttgcga atcggtctgt aacggggcaa 1380
 ggcttgaccg aggggaactat aacatgtata ggcgaaaagc ggggtctcgg ttgtaaccgc 1440
 cttaggaagt ccctctgagg tatggcagat atgcttttgc atagggaagg ggaatgtag 1500
 tcttaatcgt aggttaacat gtatattacc aaataaggga atcgctgat gcaccaata 1560
 aggtattata tgatcccat ggtggtgaag gagcgacctg agggcatatg ggcgttaaca 1620
 gaactgtctg tcttgcgtc attcctcacc ggaatcatga ccggcgagag tatgattgga 1680
 taaccaggatg gcaccattca tctggcgca tgctgattgg tgcgactaag gagtctgtga 1740

accocgaat gtacttaagc ttgtagtgc taacaataaa gtgccattct acctctcacc 1800
acattggtgt gcacctgggt tgatggccgg accgtcgatt cctcgacgac tgcgaacacc 1860
tgaatgaagc tgaaggcttc aggtaccctt acttgat 1897

<210> 11
<211> 8082
<212> DNA
<213> Human

<400> 11
agcttggttg gccgttttag ggtttgttgg aatttttttt tcgtctatgt acttgatgaat 60
tatttcacgt ttgocattac cggttctcca taggggtgatg ttcattagca gtgggtgatag 120
gttaatttcc accatctctt atgcgggtga atagtcacct ctgaaccact ttttctctca 180
gtaaactctc tttcttcgga ccttctgcag ccaacctgaa agaataacaa ggaggtggct 240
ggaaacttgt ttaaggaaac cgcctgtctc tcccccgctg gaaaccttgc acctcgagc 300
ctcctgtctc tgcctccacc tgaccocgc cctcggtgac atccaggcgc gatgatctct 360
gctgccagta gagggcacac ttactttact ttgcocaaac tgaacgcggg tgcgtcccgag 420
agagggggcg gagggaaga cgttttgag caaaatccag catagcgatt ggttgctccc 480
cgcgtttgag gcaaggcct ggaggcagga gtatttgca atccttaaa ctgaattgtg 540
cagtgcactg gatttgaag ctactatatt cacttaacac ttgaacgtg agctgocaaac 600
tcaacgggta ataaccatc ttgaacagcg tacatgctat acacacaccc ctttcccccg 660
aattgttttc tcttttgag gtggtggagg gagagaaaag tttacttaaa atgcctttgg 720
gtgagggacc aaggatgaga agaattgttt ttgtttttca tgcctgggaa taacacaaaa 780
taaaaaatcc cgagggaata tacattatat attaaatata gatpatttca gggagcaaac 840
aaatcatgtg tggggctggg caactagctg agtcgaagcg taaataaaat gtgaataaac 900
gttttcgggt tacatacagt gcactttcac tagtattcag aaaaaattgt gagtcaagta 960
actaggaat taatgccttg aaggcagcca aattttaatt agctcaagac tccccccccc 1020
ccccaaaaa aggcacggaa gtaatactcc tctctcttc ttgatcaga atcgatgcat 1080
tttttgcga tgaccgcatt tccaataata aaaggggaaa gaggacgtcg aaaggaatta 1140
aacgtccggt ttgtccgggg aggaaagagt taacgggttt ttccacaagg gtctctgctg 1200
actcccccg ctcggtccac aagctctcca ctgccccct ttagggaagtc cgggtcccgcg 1260
gttcgggtac cccctcccc tcccatattc tcccgctag cacptttgat ttctcccaaa 1320
cccgcgagcc cgagactgtt gcaaacccgc gccacagggc gcaaggggga tttgtctctt 1380
ctgaaacctg gctgagaat ttgggaactcc gtgtgggagg cgtgggggtg ggacggtggg 1440
gtacagactg gcagagagca ggcaacctcc cctctcgccct agcccaagctc ttggaacaggc 1500
agacacatct cagggtcaaa cagacgcctc cgcacagggg cccacaggaa gcctgagcag 1560
gcggggcagg agggggcgga tctgctgctt tggcagcaaa ttgggggagc cagtctgggt 1620
ggaaggtatc caatccagat agctgtgcat caataatgca taatacatga ctccccccaa 1680
caaatgcaat gggagtttat toataacgcg ctctccaaat atacgtggca atcgctgtgt 1740
gggttatatt aatcattcta ggcacgttt tctctcttat gcctctatca tctctcccta 1800
tctacactaa catccacgc tctgaacgcg cgcaccttaa taccctctt tctctcaatc 1860
tccctgggac tcttgatcaa agcgcggccc tttcccaagc cttagcgagg cgcctgcaag 1920
cctggtacgc cgtgtggcgtg gcggtggggc cgcagtgctg tctctgtgtg gagggcagct 1980
gtccgcctg cgatgattta tactcacagg acaaggatgc ggtttgtcaa acagtactgc 2040
tacggagagc cagcagagaa agggagaggg tttagagggg agcaaaagaa aatgtaggc 2100
gcgcgtatgt aattcatgct gctctcttac tctggttaca tcttagagct agagtgtctg 2160
gtgcgcggc tgagctctct cccacacctc ccaacctccc cataagcgc 2220
cctcccggtg tcccaaaagca gaggcggtg gggaaaaagaa aaagatctct ctctcgctaa 2280

tctccgccca ccggcccttt ataatgcgag ggtctggacg gctgagagacc cccgagctgt 2340
 gctgctcgcg gccgccaccg ccgggccccc gccgtccctg gctcccctcc tgcctccaga 2400
 agggcagggc ttctccagag cttggccgga aaaaagaccg agggagggat cggcctgagt 2460
 ataaaagccg gtttccgggg ctttatctaa ctgcgtctag taattccagc gagaggcaga 2520
 gggagcgagc gggcgcccg ctaggggtga agagccgggc gagcagagct cgctcgccgg 2580
 cgtctcgga agggagatcc ggagcgaata gggggcttcg cctctggccc agccctcccg 2640
 ctgatcccc agccagcggg ccgcaaccct tgcgcgcatcc acgaaacttt gcccatagca 2700
 gcggcgccgc accttgcatc ggaacttaca acaccgcagc aaggacgcga cctcccgac 2760
 gcggggagcg ttctctgccc atttggggac acttcccgcg cgtgccagg acccgtctct 2820
 ctgaaaggct cctcttgca ctccttagac gctggaattt ttctgggtag tggaaaaaca 2880
 ggtaagcacc gaagtccatc tgccttttaa ttatttttt tatcacttta atgctgagat 2940
 gagtccaatg cctaaatagg gtgtcttttc tccattctct gcgtatttga cactttttct 3000
 agagttagtta tggtaactgg ggctgggggt gggggtaact cagaactgga tcggggtaaa 3060
 gtgacttgct aagatgggag aggaagaagg agagggaaaa cgggaatgggt ttttaagact 3120
 accctttcga gatttctgcc ttatgaatat attcacgtg actcccggcc ggtcggacat 3180
 tctcgttta ttgtgttaat tgctctctgg gtttgggggg gctggggggt cgtttcggtg 3240
 gggcagaaag cccttgcat cctgagctcc ttggagttag gaccgcgtg ccctgtgtg 3300
 agccagatcg ctccgcagcc gctgacttgt ccccgctccc gggagggcat ttaaaattcg 3360
 gctcaccgca ttctgcagc ccggagacgg acactcgccg cgctcccgcc ccgctgtccc 3420
 cgcgcgcttt ccaaccgccg ctgactcttt taagaagtgt gcaattggct ttttaaaaag 3480
 caataataca atttaaaacc tgggtctcta gaggtgttag gacgtggtgt tgggtaggcg 3540
 caggcagggg aaaaaggagg cgaggatgtg tccgattctc ctggaatcgt tgacttggaa 3600
 aaaaccaggg ccgctccgc acccagccct cactcccgt ccgcggcgcc cctcggtgtg 3660
 cctcgccccc gagatgcgga ggaactgcga ggagcggggc tctggggcgt tccagaacag 3720
 gctctacct ttgtgggggt gctccggggg aggtatcgca gcggggctct cggcgagtt 3780
 gcatctccgt attgagtgcg aagggaggtg cccctattat ttttgacac ccoccttga 3840
 tttatggagg ggtgttaag cccgcggctg agctgccacc tccagccgcg gagagaaaga 3900
 agaaaagctg gcaaaaggag tgttgagcg ggcgggtact ggggtgggg agcgggggcg 3960
 tggagagggg aggttgggag gggtcggtg gccggcgggg gtaggagagc agctatgacc 4020
 cgagtgggaa cagccgcagc ggagggggcc cggcgcggag cgggggttcc gcagccgcta 4080
 gcgcaccagg gccctctgcc ttctccttca ggtggcgcaa aactttgtgc ctltgatttt 4140
 ggcaaatgtt ttctccacc gccacctccc cggtcttctt agggcgccca gggccgattt 4200
 cgattctct cccgctgcgg gcccgactcc cggtcttgc gctccgggct cccgggggag 4260
 cgggggctcg cggggcacca agccgctggt tcaataagt cgtctccgag atagcagggg 4320
 actgtccaaa ggggtgtgaa ggggtgctcc ttatttccc caccagacc acccagcccg 4380
 tttaggggat agctctgcaa ggggagaggt tgggactgt ggcgcgact gcgcgctgcg 4440
 ccaggtttcc gcaaccaagc cctttaaact caagactgcc tcccgtttt tgtgccccc 4500
 tccagcagcg tcccgcagc atgccccca cacttagctt caccacagag aactatgacc 4560
 tgcactacga ctcggtgcag cgttatttct actgcagca ggaaggagaac ttctaccagc 4620
 agcagcagca gagcagagct cagcccccgg cgcgccagca ggatatctgg aagaaattcg 4680
 agctgctgcc caecccgccc ctgtcccta gccgcctctg cgggctctgc tgcctctct 4740
 acgttgcggt caacaccttc tcccttcggg gagacaacga cggcggtggc gggagctct 4800
 ccacggcgca ccagctggag atggtagcgg agctgctggg agggagacat gtgaaccaga 4860
 gttctactcg cagcccgga gacgagacct catcaaaaa catcatcatc caggactgta 4920
 tgtggagcgg ctcttcggcc gccgccaaag tctgtctaga gaagctggcc tctaccagg 4980
 ctgocgcgcaa agacagcggc agcccgaacc ccgcccggcg ccacagcgtc tcttccacct 5040
 ccagcttgta cctgcaggat ctgagcgccg ccgctcaga atgctcgac cgcctggtgg 5100
 tcttccccta cctctcaac gacagcagct cgcaccaagt ctgcgctcg caagactcca 5160

ggcgcctcttc tccgtctctcg gattctctgc tctcctcgac ggagtcctcc ccgcaggcca 5220
 gccccgagcc cctgtgtctc catgaggaga caccgcccac caccagcagc gactctggta 5280
 agcgaaagcc gcccaggcct gtcaaaagtg ggcggtgga tacctttccc attttcattg 5340
 gcagcttatt taacgggcca ctcttattag gaaggagaga tagcagatct ggagagattt 5400
 gggagctcat cacctctgaa accttggggt tttagctttc ctcccatccc ttccccttat 5460
 actgcctcatg ttctcagccc cctcccctgt ttgtctccca cccctcagga atttcaattta 5520
 gggtttttaa ccttctggct tatcttaaca ctcaatccac ttcttcttac ctcccgttaa 5580
 cattttaatt gccctggggc ggggtggcag ggagtgtatg aatgaggata agagaggatt 5640
 gatctctgag agtgaatgaa ttgcttccct cttaacttcc gagaagtggc gggattttaa 5700
 gaactatcta caaaaatgag gggtctgttt tagaggctag gcaggcgctg cctgagtgcg 5760
 ttgagccagt aactgcctca agagtgggtg ggctgaggag ctgggatctt ctgcagctat 5820
 ttgaaacctg gaaaagcaaa tccttgccaa agttggactt ttttttttct tttatttctt 5880
 ccccccgcct cttggacttt tggcaaaact gcaatttttt tttttttatt tttaatttcc 5940
 agtaaaatag ggagtgtcta aagtcatacc aagcaatttg cagctatcat ttgcaaaccc 6000
 tgaagtgttc ttgtgaaagt cctcaaaaa ttggaggatg ttgggaatgt gctttgtctt 6060
 ggggtgtgcc aaagcctcat taagtcttag gtaagaattg gcatcaatgt cctatcctgg 6120
 gaagtgtcac ttttctgttc catgccataa cccagctgtc ttccctttta ttgagacttt 6180
 acctcatgg ttgagggagt aaagggtggct ggctagattg gttctttttt ttttttttcc 6240
 ctcttttaag acggagtctc actctgtcac taggctggag tgcagtggcg caatcaacct 6300
 ccaaccctct ggttcaagag attctctgc ctcagcctcc caagtagctg gactacagg 6360
 tgcacaccac catgccaggc taatttttgt aatttttaga gagatggggt ttcatcgtgt 6420
 ttggccaggat ggtctctctg gacctcacga tccgcccacc tcggcctccc aaagtgtctg 6480
 gattacaagt ttgagccagg gccaccagct tagatgtggc ttttggggcg gataattttg 6540
 tccagagacc ttctcaactg attcatgctt tgtattttga cagcattaat ctggtaattg 6600
 attattttta ttgaaccttg ctaaaaggag gatttctatt tcctttctta aagaggagga 6660
 caaagaagat gaggaagaaa tcatgttgtt ttctgtggaa aagaggcagg ctctcgcaa 6720
 aaggctcagag tctggatcac ctctctgttg aggccacagc aaacctctcc acagcccact 6780
 ggtctctcaag aggtgccacg tctccacaca tcagcacaac tacgcagcgc ctccctccac 6840
 tcggaaggac tatcctgctg ccaaggaggt caagtggag agtgtcagag tctgagaca 6900
 gatcagcaac aaccgaaaat gcaccagccc caggtctctg gacaccgagg agaattgcaa 6960
 gaggcgaca cacacgtctt tggagcgcca gaggaggaac gagctaaaa ggagcttttt 7020
 tgccctgctg gaccagatcc cggagtgtga aaacaatgaa aaggcccca atgctgttat 7080
 ccttaaaaaa gccacagcat acatctctgc cgtccaagca gaggagcaaa agctcatttc 7140
 tgaaggagag ttgttcgga aacgacgaga acagttgaaa cacaaaactg acacagctag 7200
 gaactcttgt cgtaaggaa aagtaaggaa aacgattctt tctaacagaa atgtctcag 7260
 caatcaccta tgaacttgtt tcaaatgcat gatcaaatgc aacctcaca ccttggtcga 7320
 gtcttgagac tgaagattt agccataatg taaactggct caaattggac ttggggcata 7380
 aaagaacctt ttatagatt ccatcttttt ttttctta acagatttgt atttaagat 7440
 tgtttttaa aaattttaag atttacacaa tgtttctctg taaatattgc cattaaatgt 7500
 aaataacttt aataaaactg ttatagcagt tacacagaat ttcaatccta gtatatagta 7560
 cctagtatta taggtactat aaacctaat ttttttatt taagtacatt ttgcttttta 7620
 aagttgattt ttttctattg ttttagaaa aaataaaaa actggcaaat atatcattga 7680
 gccaaatctt aagttgtgaa tgttttgttt cgtttcttcc cctcccaac caccaccatc 7740
 cctgtttgtt ttcatcaatt gcccttcag agggcggtct taagaaagc aagatttttc 7800
 ctctgttgaa atgggtctgg gggccttaag gtctttaagt tcttgagggt tctaagatgc 7860
 ttctctggaga ctatgataac agccagagtt gacagttaga aggaatggca gaaggcaggt 7920
 gagaaggtda gaggtaggca aaggagatac aagggtgcaa aggtgacagt taagtacaca 7980
 aagaggcata aggactgggg agttggggagg aaggtgagga agaaactcct gttacttag 8040

ttaaccagtgc cagctccct gctcactcca aaccaggaa tt

8082

<210> 12

<211> 4480

<212> DNA

<213> Rat sarcoma virus

<400> 12

agggttacac gtcttaactc agagttgcac caggcttgaa caagcccagg cagcccccaga 60
 tacctaggcg caggtcacgc ttaaaactaa cagaccataa aaggaagagg ataccagaga 120
 gactaggagt accggatctg actcacaggc cactctggcg gaagagataa gccccagccc 180
 ccgacattca ggacgtccca gcccgacgt actcttacca tgtttacacc tcttcgaat 240
 atgattcaaa cctgccaaag tgtgtagcta taccttatca cctcatcttg tgaataaacc 300
 aatcatatgt gaacatgtct atatgcttcg tttaaatcca ccaatccccc taactatgca 360
 tctgtctctg tacgcgcgct tctgtctccc caaacccat aaaagcccca tgcctagagct 420
 gtgtggcgcg caagtccctc gaagagactg tgtgcccgca ggtacctgtg ttttccaata 480
 aacctcttg cgtgtgcat ccgagtgggc tcggtctcgt cactgggcgc ttgggggtct 540
 cctectgagg gaaaggtcct ctccggaggt cttttcatt ttgggggctg tccgggatct 600
 ggagatctcc cgcccagaga tcaccgacca cccaccggga ggttaagccg ccggcatctg 660
 tctgtctctg cctgtctctg tctgtctctg tctgtgcgc gtgttcagtt cgtctcagtt 720
 ttggaactca atctgggttt tggtcgaagg agaaggccca gggcttcggt tcttcagggt 780
 ctaggacctc cagcgctccc gtttggcggt gtcagagaag gagctgacga gctcggactt 840
 ctccccccgc agccctggaa gacgttccaa ggggtgtctg agccccgttc ttggggctc 900
 agcccgatc ggaggggatac gtggttttgg ttggaggaga gggctccagg cctctggcac 960
 ctccatctga ctctttgttt tgggttttcc gtcgaagccg cgcggcgctg ctgtctgtta 1020
 tttgtctgat cgttggattt gctgtcttaa tctgtccct aattttcttt gaagctacca 1080
 tgggacaatc gctaacaacc cctttagctc tcaactctga ccatgtgaag gacgtccgag 1140
 accgagcagc tgatcagctg gtcgagatca agaaaggctc tctccggagg tcggggagag 1200
 tcgcgccagc aagcgtgtgg gcaggagctc ctggtttggc agccctgtga gaagctgga 1260
 cagaatacaa gcttgtggtg gtggcgcgta gaggcggtgg aaagagtgc ctagaccatc 1320
 agctgatcca gaaccatttt gtggacaggt atgatccac tatagaggac tctaccgga 1380
 aacaggtagt cattgatggg gagacgtgtt tactggacat cttagacaca cgaggtcaag 1440
 aagagtatag tgccatgcgg gaccagtaca tgcgcacagg ggagggcttc ctctgtgtat 1500
 ttgccatcaa caacaccaag tcctttgaag acatccatca gtacagggag cagatcaagc 1560
 ggggtgaaaga ttcagatgat gtgccaattg tctgtgtggg caacaagtgt gactgtggcg 1620
 ctacacactgt tgagtctcgg caggcccgag acctgtctcg cagctatggc atccctaca 1680
 ttgaaacatc agccaagacc cgaccaggtg tggaggatgc cttctacaca ctagtacgtg 1740
 agattcggca gcataaactc cggaaactga acccgctcga tgagagtggc ctggtctgca 1800
 tgagctgcaa gtgtgtgctg tctgacacc aggttaagga cctgatttct cgccagaagc 1860
 cgtacggaca cctgaccag gtggccctca ttgtcacctg ggagagcttg gcatttagcc 1920
 ctctctctg ccgcagaacc ttgtgggacc cgaattggct tctgtttccc cctaaacctg 1980
 ttcccccgag cccacctgac cctttgtgtg ctcttctccc tctctatcct gctctaaacta 2040
 aggaagaatc tcccaaagtc cctcccccca aacctgtcct cccagaggac ccaaatcccc 2100
 ccttataga tctcctgttg gaagaacctc ctccgtaccc tgaactaca gccccgccaa 2160
 gaagaagagg agtgagagcg cctgctagac ctgactcga ggcggccctc tccctgtgtg 2220
 ctggaagact tcggggacga cgcgaggtgg cgccagactc cactcccgag gcttttcgcg 2280
 ttagacaagg gctggcgcg cagatacaat actggccatt ctacgggcc gacatatata 2340
 actggaaaca acacaacccc cctttttcta aggatccggt ggtctcacc aaccagatag 2400

aatctgtctt gcttaacccat cagcccactt gggatgatat acagcaactt ttacaggccc 2460
 tcttcgacct tgaagagaag cagagagtg cctttagaggc caggaaacat gttttggggg 2520
 acaatggagc cccccccttg ctcccgaagg agatcgatga tgcattccca cttacaagac 2580
 ctgattggga tttccaccag gctaaaggta ggagacacct acgcctttat cgccagttgc 2640
 tcttagcggg tctcggagg gcggcacgac gcccccacaa tttggctcag gtaaaaacag 2700
 tggtaacaaga ggctcgagg agtccctcag ccttcctaga gagacttaag gaagcttate 2760
 cgcgtatata cctctatgat ccagatgatc caggacaaat gacaaatgtc tccatgtcct 2820
 tcatctggca ggcagaccca gatatacagg ccaagctaca gagaatagaa aatttacaag 2880
 ggtatacact cgaggattta cttaaggagg cagaaagaat ttataacaag agagagacac 2940
 aagaagaaaa gaaagataaa atacgttagag aaaaagatga gagagaccga aaaaagaaac 3000
 gagagttagg tcgaattctg gcgcgcgttg ttcagggtca agagaaaagg ggagagaggg 3060
 tgggagttcg aaaggggcca aagctagata aggatcaatg tgcgtattgc aaagaaagag 3120
 gacactgggc cagagattgc cctaagaaac ccagcgctc cgaagacccc gccccacagac 3180
 tccccctctg cccctagata aagattaggg aggtcagggc caggagcccc cccctgagcc 3240
 caggataact cttgaagttg gggggcagcc agtcaccttt ctggtggaca caggagcccc 3300
 gcactcagtc ctacccagg ccctcggaac actcagcgac cggaacggct gggtacaagg 3360
 agccactggc agcaagagat accgttggaac tacagatcga cgggttcagc tggctactgg 3420
 taaggtgacc cattccttct tacatgttcc ggactgcccc tacctctcgc tggggcctga 3480
 ctctgcttacc aaatataaag ctcatagcca ttttgaaga ggagggagcc gatataccgg 3540
 gccccggctt attcctcttc agattttaac ccttcagtta gaagatgaat atagattata 3600
 tgaaccagaa caggacaagc caaaatctcc agaaatagac tcttgggtca cgaattccc 3660
 actggccttg gcagagactg gcgggtaggg gttggcgctc caacagcctc ccttaattat 3720
 ccagttaaag gccaccgoga ctctgtctc cattaaacag taccccatcg catggggaagc 3780
 ttatcagggc ataaagccac atatcaggag gctcttagac caaggcatcc tagtcccttg 3840
 ccggtcaccc tggaatacgc ctctgtacc tgttaagaag cccggcactc gagactatag 3900
 gccagtacaa gatttgagag aggtcaacaa aagagtagaa gatattcatt caactgtccc 3960
 aaacccttat aacctactca gcaccctgcc tcccaccat acttggtata cgttcttaga 4020
 tctgaaggat gctttcttct gcctccggct gagcccagaa agccagccct tatttgcttt 4080
 tagtggaaga gactctgaaa tggggctttc gggacagtgt acttggaacaa gattaccaca 4140
 gggtttcaaa aacagcccaa cgctctttga tgaggcctta caccgggact tggctgactt 4200
 tcgagtcagg catccactc ttatacttct tcagtttgtt gatgaccttc ttctaggggc 4260
 cactcttgag acagcatgcc accagggaac agaattccctc ttgcagactt tggggcgatt 4320
 gggctatcga gcttctgcca gaaaggctca aatttgccag acccaggtta ctattattag 4380
 ctatocacta agggatggac agcgatggct gactccggct aggaacacaga ccgtggccaa 4440
 catccagacc ccaagaaatg gccgacagct acgggaattc 4480

<210> 13

<211> 565

<212> DNA

<213> Rat sarcoma virus

<400> 13

gctgagtagt gcgcgagcaa aatttaagct acaacaaggc aaggcttgcc cgacaattgc 60
 atgaagaate tgcttagggg taggcgtttt gcgctgtctc gcgatgtacg ggccagatat 120
 acgcgtatct gaggggacta ggggtgtgtt aggcgaaaaa cggggcttcg gttgtacgac 180
 gttaggagtc cctccagat atagtagttt cgcttttgca taggggaagg gaaattagat 240
 cttatgcaat acctctgtag tcttgcacaa tgcttatgta acgatgatt agcaacatgc 300
 cttacaagga gaaaaaagc accgtgcatt ccgatttggt gaagtaagggt ggtacgatgc 360

tgcccttatta ggaaggaac agacgggtct gacatggatt ggacgaacca ccgaattccg 420
 cattgcagag atattgtatt taagtgccta gctcgataca ataaacgcca tttagaccatt 480
 caccacattg gtgtgcacct ggggtgatgg ccggaccgtt gattccctga cgactacgag 540
 cacctgcatg aagcagaagg ctcca 565

<210> 14

<211> 1804

<212> DNA

<213> Human

<400> 14

ggatcctcag gggtaacacc ttttggaggt gggcatcttc ctcattctca gtggtgccaa 60
 ttcatatcc tgcctggcta acacgtgggt ttactatatt tctggcctta tatgattatg 120
 aagctagaac tacagaagac ctttcattta agaagggtga aaaatttcaa ataattaca 180
 atacagaagg agactggtg gaagcaagat caatcactac aggaagaat ggttatatcc 240
 tgagcagtta tgtagcgct gcagattcca ttcaggcaga agaattggtat ttggcaaaa 300
 tggggagaaa agatgctgaa agattacttc tgaatcctgg aaattaatga ggtattttct 360
 taggaagaga gagtgaatg gctgggtgca gtggtctatg cctgtaatcc cagcactttg 420
 ggaggccgag ttggcggat cacctgaggt caggagtctg agactagcct ggcacaacatg 480
 gtgaacccc atctcacta aaaaaaaag tacaaaatta gctggacgtg gtggtgagtg 540
 cctgtaatcc cagctactca ggaggctgag gcagcagaat cacttgaaac tgggaggcgg 600
 aggtgtcagt gagctgagat cgcgccactg cactccagcg tggcgacaa gagcaaaaac 660
 tccgtctaaa aaacaaataa gcaaacagaa caaaacaaaa caaaacgag agagcgaaac 720
 tactaaaggt gcttattccc tctctattcg tgattgggat gaggaagggt gtgacaatgt 780
 gaaacaccac aaaattagga aacttgacaa tggtagatag tatatcacaa ccagagaaca 840
 acctgtact ctgcagaat tggcaaaaca ctacacagaa catgctgatg gttattgcca 900
 caagtaaca actgtgtgtc caactgtgaa acctcagatt caaggtctag caaaagatgc 960
 ttgggaaatc ccttgataat ctttgcact agagggttaa ctaggacaag gatgttttgg 1020
 caaagtgtg atgggaatat ggaatggaac cacaagaatg gcaatcaaaa cactaaaacc 1080
 aggtacaatg atgcagaag cttttctcca agaagctcag gtaatgaaaa aaataagaca 1140
 tggtaacct gtccactat atgctgttgt ttctgaagag ccaatttaca ttgtcactga 1200
 attgatgtca aaaggaaagt tattcaattt ccttaaggaa ggagatggaa agattttgaa 1260
 gttccacaa atggttgata tgcctgtcca gattgctgat ggtatggcat atattaaaag 1320
 aatgaactat attcaccgag atctctgggc tgctaattat cttgtaggag aaaattctct 1380
 gtgcaaaata gacagttttg gtttagcaa gttlaattga gacaatgaat acacatcaag 1440
 acaaggtgca gaattcccaa tcaaatggac agctcctgaa gttgactgt atggtgggtt 1500
 tacaataaag tctggtgtct gctcatttgg aattctacag acagaactgg taacaaaggg 1560
 cagagtgcga tatccaggtg tggtagaaca tgaaatactg gaacaggtgg agcgagga 1620
 caggatgctt tgccctcagg gctgtccaga atccctccat gaattgatga atctgtgtt 1680
 gaagaaggac cctga-gaaa gaccaacatt tgaatatgtt cagtcctct tgggagacta 1740
 cttcactgct acagagccat agtaccagcc agggagaaaac ttctaattca agtagccat 180C
 tttta 1804